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13. ABSTRACT (Maximum 200 Words)

Systematic drugs are one of the most potent means for controlling breast cancer. Their ability to kill cancer cells often depends, however, on the presence of appropriate physiological conditions. For instance, exciting recent results suggest that uptake and retention of 5-fluorouracil (5-FU) in tumors may be influenced by pH, in particular, the trans membrane pH gradient. We designed novel_^{19}F NMR reporter molecules to investigate breast tumor pH. Several syntheses of novel molecules have been successful (e.g., trifluoromethyl pyridoxol) and he molecules have been characterized in vitro and evaluated in vivo. Certain strategic molecular goals proved to be intractable, but have provided valuable lessons in synthetic chemistry. The new indicators have provided non-invasive measurements of extra cellular pH in breast tumors, which is an important step towards understanding factors modulating chemotherapy. This research also laid a foundation for a novel concept in gene therapy, ^{19}F NMR reporter molecules based on the fluoronitrophenol galactoside structure.

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Introduction

Systemic drugs are one of the most potent means for controlling breast cancer. Their ability to kill cancer cells often depends, however, on the presence of appropriate physiological conditions. For instance, exciting recent results suggest that uptake and retention of 5-fluorouracil (5FU) in tumors may be influenced by pH, in particular, the trans membrane pH gradient. This opens the possibility of predicting which tumors will show best response by measuring pH *a priori* or modulating tumor physiology to optimize tumor selectivity. We developed a novel class of non-invasive NMR pH indicators and propose a novel second generation of enhanced indicators for investigating breast tumor pH. Specifically, we proposed to survey a series of diverse breast tumors in order to examine the specific correlation of 5-FU uptake versus pH gradient. In addition, we proposed to investigate the feasibility of manipulating pH in order to enhance breast tumor uptake.

We had three specific aims for this project:

Phase 1 Design, synthesis and evaluation of next generation molecules:
Based on our experience with 6-fluoropyridoxamine (6-FPAM) (1, 2), we designed a second generation of enhanced pH indicator incorporating a trifluoromethyl group. This was predicted to provide three times greater signal intensity, improving the precision, and accuracy, of pH measurements *in vivo*. We proposed to synthesize and characterize this new generation of improved ^{19}F NMR pH indicators.

Phase 2 Evaluation of a novel series of pH indicators *in vivo*:
Test trifluoromethyl pyridoxol in breast tumors *in vivo*. We expected the proposed pH indicators incorporating CF_3 reporter groups would offer considerable advantages over existing pH indicators. These fluorine NMR pH indicators exhibit NMR chemical shift response to changes in pH. Preliminary data had suggested that they would readily permeate cells providing simultaneous measurement of intra- and extracellular pH, and hence, the pH gradient.

Phase 3 Application of the new molecules to critical issues in breast cancer:
pH was measured in breast tumors implanted in rats and mice. Measurements were compared with traditional polarographic electrodes. Since pH may regulate the biodistribution and retention of chemotherapeutic drugs, such as 5-fluorouracil, the ability to measure tumor pH and detect changes in the pH was tested.

Body

During the four year duration of this project (thee years funded plus 1 additional year of no additional cost extension) most of the original tasks (SOW) were tackled and many completed successfully. Unexpected difficulties with detecting sufficient ^{19}F NMR signal from tumors *in vivo*, lead to curtailment of certain tasks. Two organic synthetic chemists received training in the development of fluorine labeled molecules and their applications to ^{19}F NMR. Trifluoromethyl pyridoxols were successfully synthesized and found to exhibit enhanced signal per unit mass. PH measurements were undertaken in breast tumors in rats and mice using the novel ^{19}F NMR pH indicators together with validation based on traditional electrodes. ^{19}F NMR pH indicators and the popular chemotherapy drug 5FU could be detected simultaneously in breast tumor, however signals were quite weak and transient. As expected CF_3-POL provided enhanced signal to noise over first generation indicators (6-fluoropyridoxol (FPOL) and 6-fluoropyridoxamine (FPAM)), but the chemical shift response to pH changes was much smaller. A primary synthetic target was di-CF_3-pyridoxol, which was expected to improve chemical shift sensitivity and provide an internal chemical shift calibration standard, but this class of molecule proved elusive. Nonetheless, study lead to the discovery of a novel class of pH reporter molecule (fluoronitrophenols) and a novel concept – that of ^{19}F NMR sensitive gene reporter molecules. Fluorophenyl galactosides are now being developed to detect the efficacy of gene therapy based on the expression of β-galactosidase, the product of the lacZ gene. Detailed results for each specific task in the original statement of work are provided below, with additional results and manuscripts in the appendices.

Statement of Work

Phase 1 Design, synthesis, and evaluation of enhanced ^{19}F NMR pH indicators:

Task 1 Recruit and train post-doctoral fellow in synthesis of fluorinated vitamin B6 analogs
Dr. Pieter Otten was recruited in year 1 to undertake molecular syntheses. He developed impressive skills in synthesis of fluorinated vitamin B6 analog pH indicators. However, he was given an outstanding opportunity to join Chembridge Pharmaceutics in San Diego, where he now leads a research team. We were fortunate to recruit a second outstanding organic synthetic chemist, Dr. Jianxin Yu, who also demonstrates exceptional synthetic skills.
Task completed

Task 2 Synthesize CF_3-pyridoxol
CF_3-POL was successfully synthesized, as presented at several conferences (Appendix 1). Manuscripts are in preparation and a draft is included in Appendix 2. Synthetic route and yields are shown in Fig. 1
Task completed

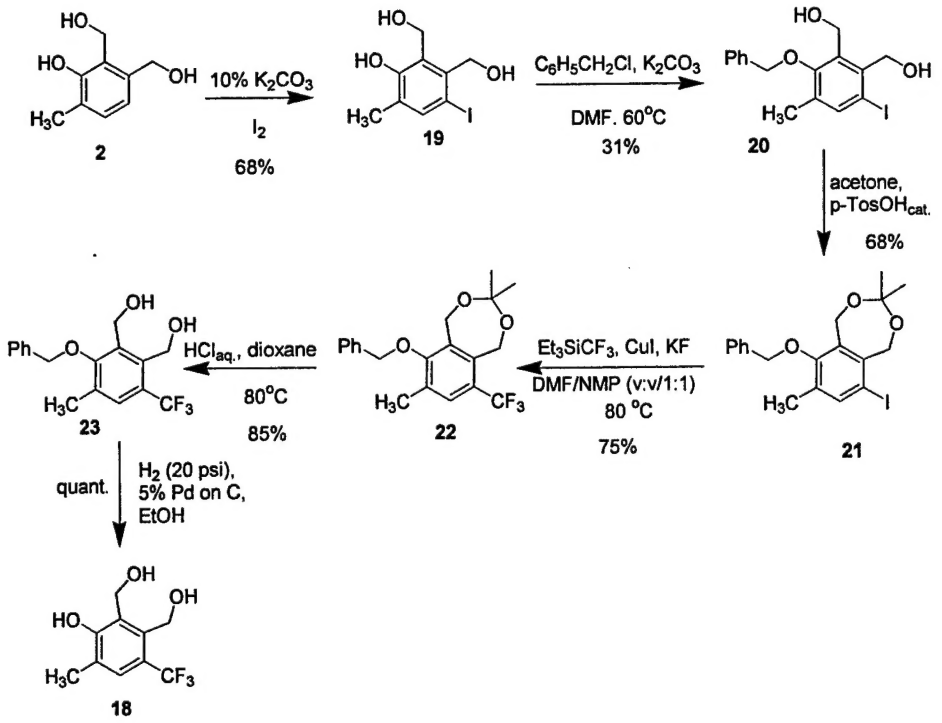


Figure 1

Task 3 Synthesize CF_3-pyridoxol derivatives modified at 4, 5 and 2 position Derivatives of CF_3-POL were successfully synthesized with characteristics presented below and further details provided in Appendix 3. In addition to the successful syntheses, a major synthetic effort was applied to synthesizing di-CF_3-POL (Appendix 4). Results of synthetic strategies and outcomes are provided.
Task completed

Since synthesis of CF_3-POL is technically more challenging than FPOL, we first investigated the introduction of a chemical shift calibration group on the simpler structure. The first target using a SCF_3 moiety proved very poorly water soluble (Figure 2).

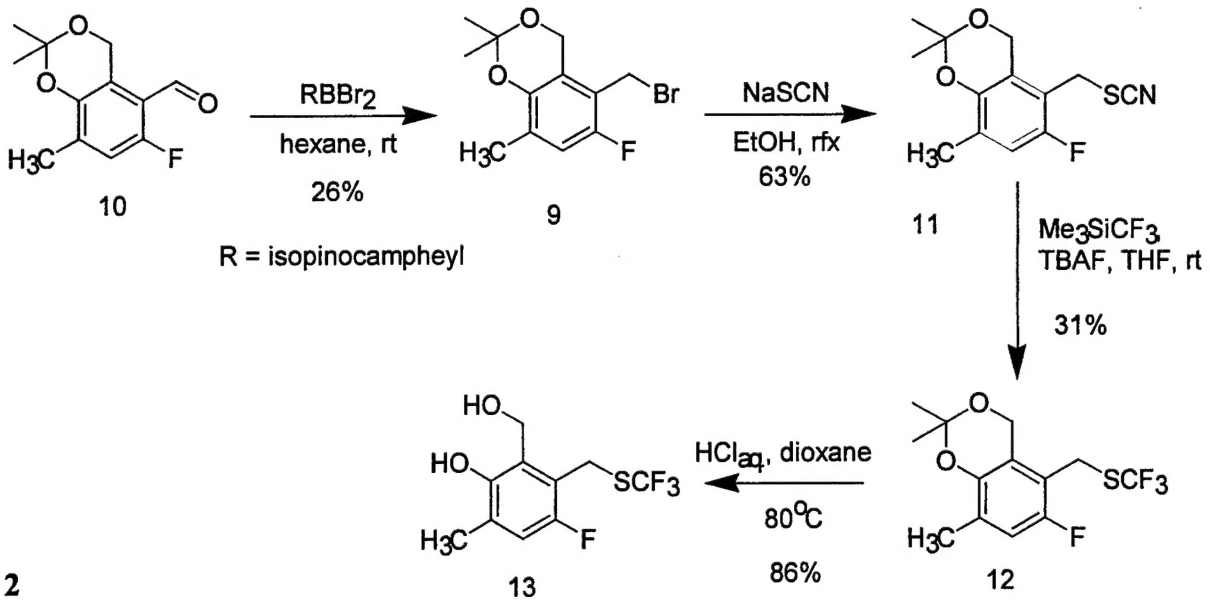


Figure 2

As an alternative we synthesized a trifluoroacetamide. Synthesis was successful (Figure 3) with good yields. However, NMR showed two peaks (Task 4 below) which are attributed to isomers. As such this approach was not pursued with the CF_3-POL.

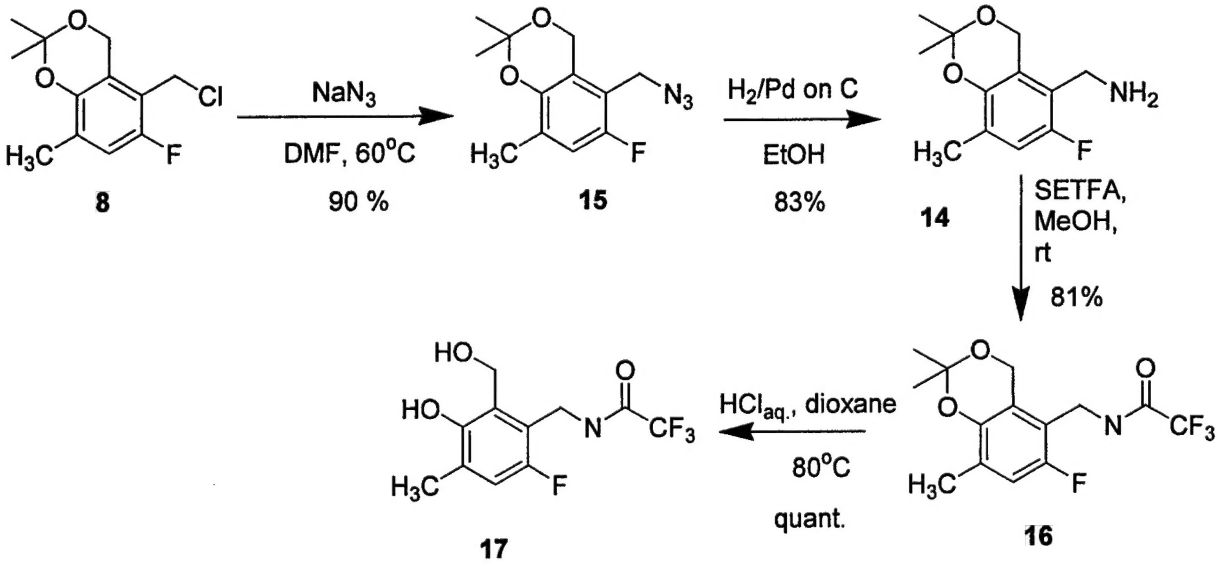


Figure 3

As described below CF_3-POL was found not to enter cells. We thus undertook syntheses of alternative molecules which were designed to enter cells based on literature reports, *e.g.*, Figure 4 shows synthesis of sugar derivatives incorporating glucose or mannose which were expected to enter cells. While testing ^{19}F NMR signals were obtained, we have not yet found any evidence for cellular penetration.

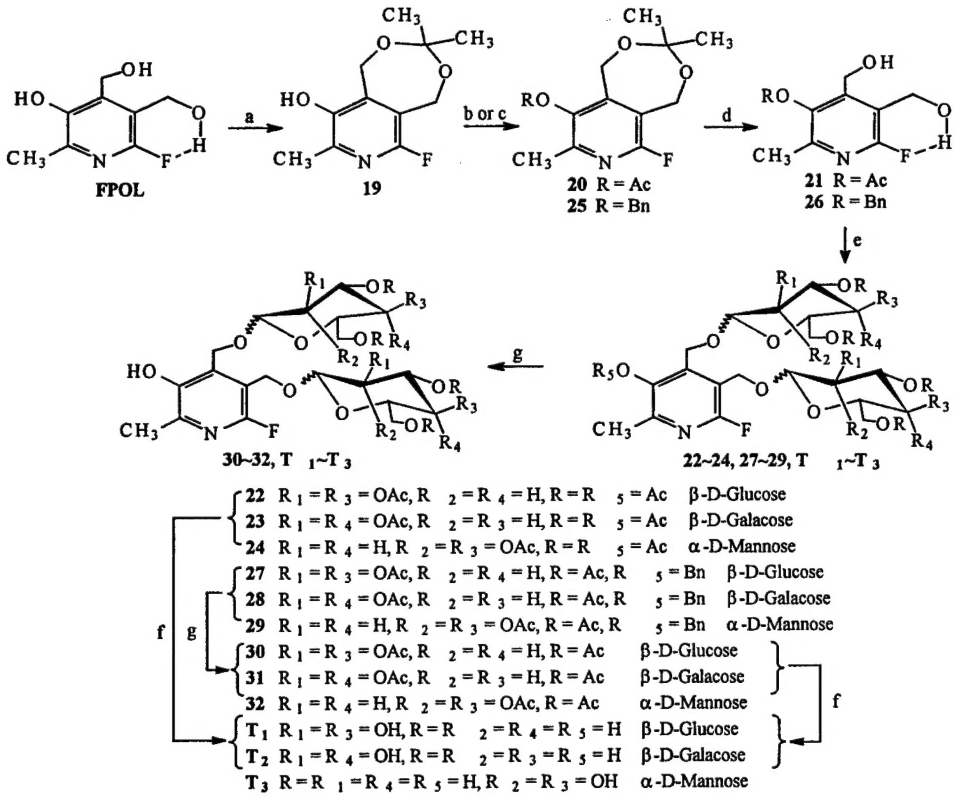


Figure 4.

A major goal was synthesis of di-CF_3-POL. This sequence shows our primary synthetic approach

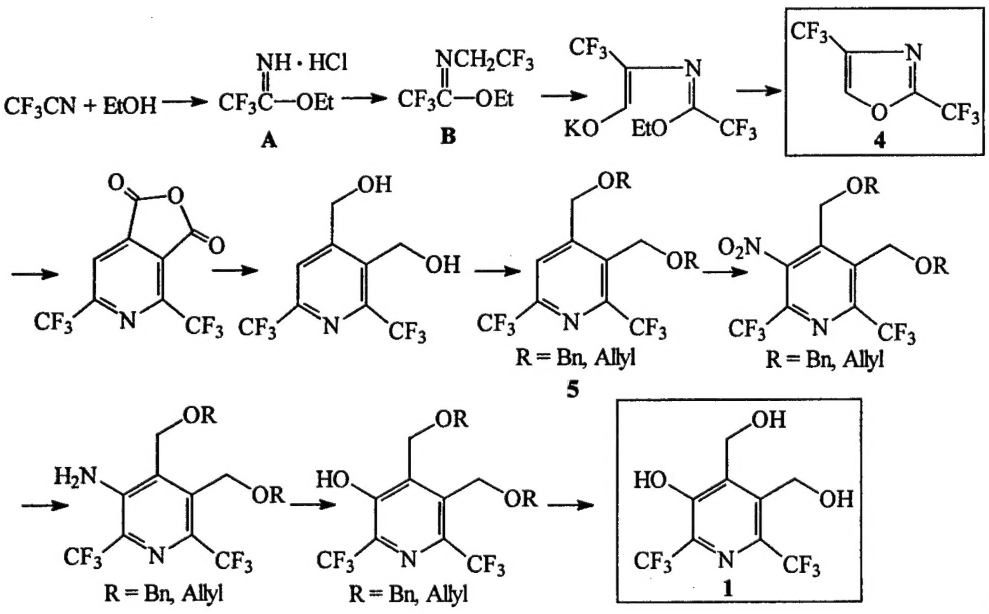


Figure 5

Unfortunately the di CF_3-POL proved intractable. Further details are provided in Appendix 4.

Task 4 Characterize new pH indicators, *e.g.*, ^{19}F NMR titrations, high-resolution mass spectrometry, ^{1}H, ^{19}F and ^{13}C NMR structural analysis

The CF3-POLs were characterized in terms of structure and ^{19}F NMR titration curves, as shown in Table 1 and Appendices 1 & 2. While exploring synthetic routes, we discovered a novel class of potential NMR reporter molecule based on fluoronitrophenol. Since these were commercially available and potentially avoided difficult syntheses, molecules were characterized and titrations performed. These results provide a database to assist in evaluating chemical, structural, and spectral characters useful for a ^{19}F NMR pH indicator.

Task completed.

Table 1

Molecule	**δacid**	**δbase**	**Δδ**	**pKa**	**comments**
CF_3-POL	15.1	16.7	1.64	6.82	extra cellular only
FPOL	-9.85	-19.61	9.7	8.2	
FPAM	-9.19	-19.19	10.1	7.05	
FPOL-SCF_3					very poorly soluble
FPOL-$NCOCF_3$			(11.1 + 11.5)	(7.7 + 8.1)	two signals- isomers
PFONP	-46.44	-55.75		6.85	cytotoxic
p- trifluorophenol	-14.44	-15.68	1.25	8.5	
o-trifluoromethyl-phenol	13.63	14.03	0.4	7.9	
2-nitro-4-trifluoromethyl-phenol			~1	5.46	2 signals
4-nitro-3-trifluoromethyl-phenol			~0.5		complex signals
2-chloro-5-trifluoromethyl-phenol	13.09	13.38	0.29	6.5	split signals

all chemical shifts quoted in ppm relative to sodium trifluoroacetate at 0 ppm

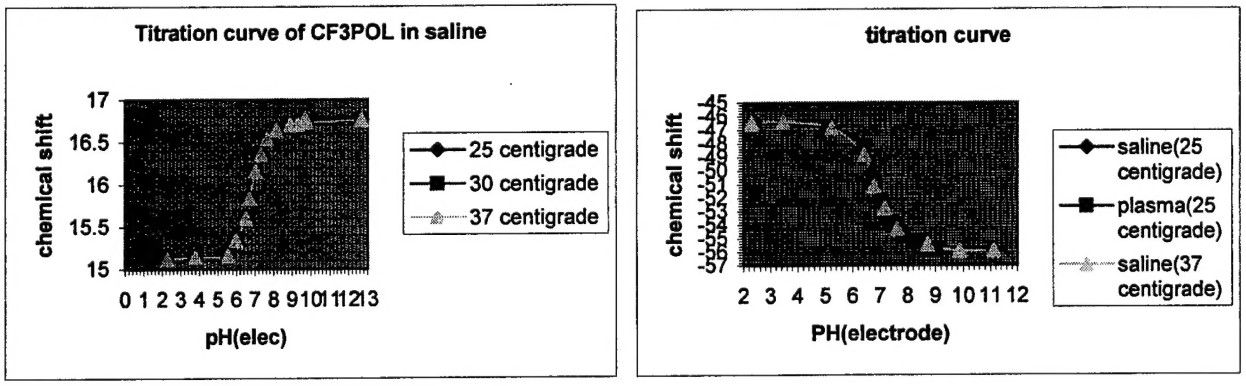
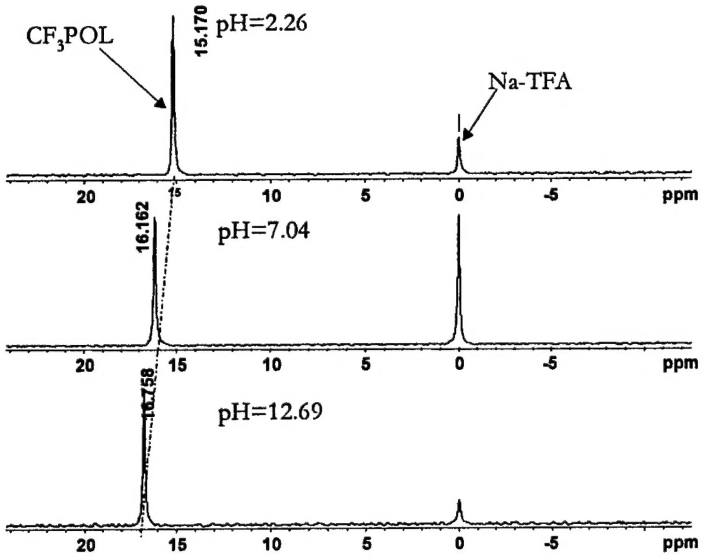
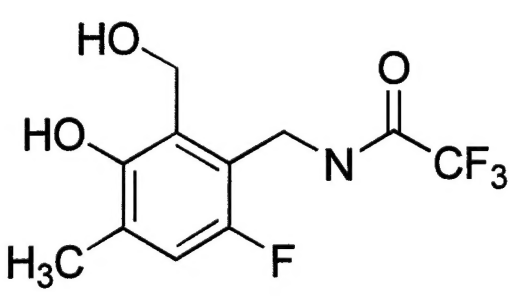


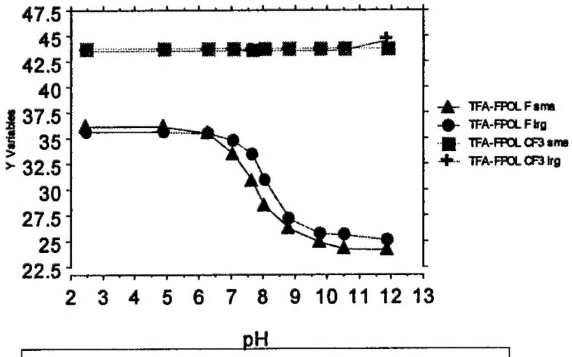
Figure 6 Titration curves for CF_3POL (left) and PFONPG (right)

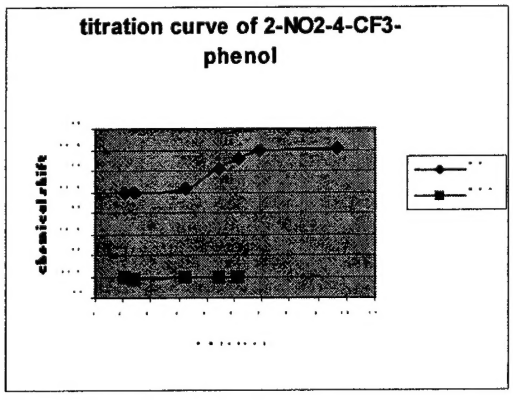


- **CF_3POL in saline, Na-TFA as a external reference**

Figure 7







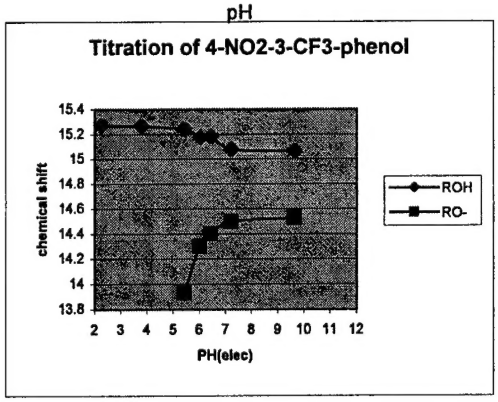


Figure 8

Task 5 Evaluate molecules in plasma and whole blood

Titration curves were obtained in saline and plasma for CF_3-POL and several other candidate pH indicators, as well as in whole rabbit blood. CF_3-POL gave a single sharp ^{19}F NMR resonance in each case showing that acid and base forms were in fast exchange on the NMR time scale at 4.7 and 9.4 T. Titration curves were identical in saline, plasma and at various temperatures. PH measurements were validated using electrodes, ^{31}P NMR, and alternate ^{19}F NMR pH indicators such as the first generation 6-FPOL. CF_3-POL showed only a single resonance in whole blood indicating lack of cellular penetration. Other molecules showed two signals in whole blood indicating cellular uptake and the ability to measure transmembrane pH gradients. Typical spectra and titration curves are provided and further details presented in Appendix 4.

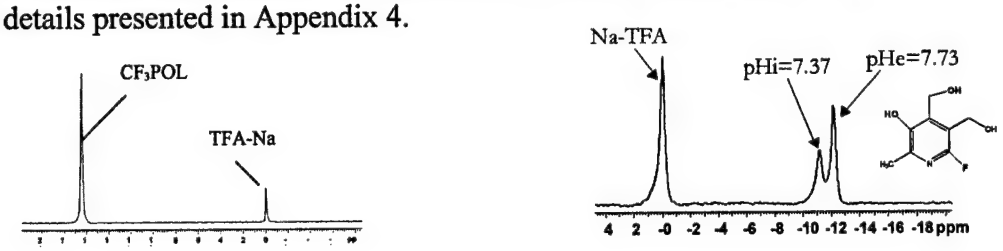


Figure 9 Left: CF_3POL and right FPOL in whole blood

Para-fluoro-ortho-nitrophenol (PFONP) represents a novel class of pH sensor molecule. The single ^{19}F NMR signal exhibits a chemical shift range of 9.3 ppm between acid and base and pKa = 6.85. Upon addition to whole blood, two signals attributed to intra- and extra-cellular compartments were rapidly observed, *e.g.*, –52.68 and –54.21 ppm corresponding to pHi = 7.16 and pHe = 7.55.
Task completed.

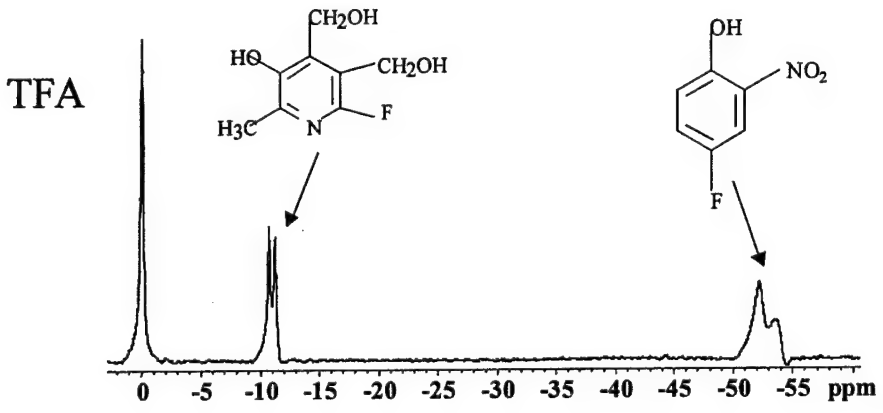


Figure 9 Comparison of transmembrane pH gradients assessed in whole rabbit blood by FPOL and PFONPG

Task 6 Scale up synthesis of most promising molecules

CF_3-pyridoxol synthesis was scaled up to provide sufficient for *in vivo* evaluation. Additional indicators such as PFONPG can be obtained in large quantities from commercial sources such as Aldrich.

Task completed.

Task 7 Evaluate most promising molecules in perfused heart model

CF_3-POL and PFONPG were evaluated in perfused heats. Spectra shown in Figure 11. In blood and perfused hearts, CF_3-POL gave a single signal only, verifying lack of cellular penetration. There was no overt acute toxicity. However, PFONPG caused rapid cessation of mechanical heart activity such suggesting sever acute toxicity, presumably by ionophoric depolarization, by analogy with the classic uncoupler dinitrophenol. The observation of toxicity has since led to successful application to the DOD prostate cancer initiative to support a molecular biologist to investigate novel gene activated broad-spectrum chemotherapy. It had been hoped that the fluorinated phenols would provide useful indicators of pH gradients

Task complete.

Figure 11 Left CF_3POL in perfused heart; right FPOL in perfused heart

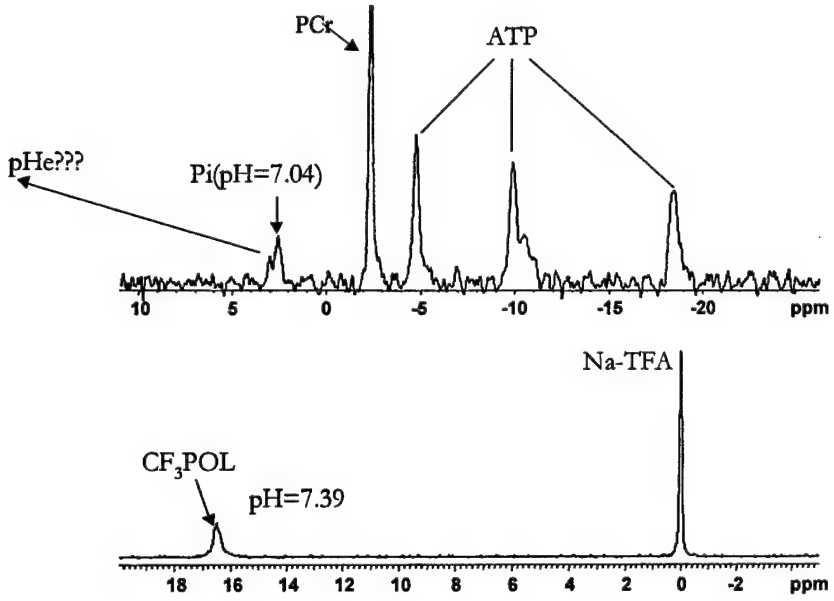


Figure 12. In a perfused heart transmembrane pH gradient could in principle be detected from intra and extra cellular Pi. However, overlap may require deconvolution reducing the certainty in measurements. By contrast the CF_3-POL, which gives a single extra cellular signal only, can have advantages for estimating extra cellular pH alone.

Task 8 Prepare reports and manuscript.

Report was provided and draft manuscripts were prepared.
Task complete.

Phase 2 Evaluation of optimal pH indicator *in vivo*:

Task 9 Evaluate best molecule in breast tumor subline 13762NF

An additional post doc (Dr. Weina Cui) with expertise in chemistry and pharmacology was recruited and trained in NMR and small animal husbandry. Breast tumors were prepared and ^{19}F NMR studies undertaken *in vivo* in both control and tumor bearing rats. Four agents were tested. CF_3-POL the target of this project, together with previously reported pH indicators, since CF_3-POL showed lack of cellular uptake. Some tests showed a lack of ^{19}F NMR signals in tumors *in vivo*. Additional tests were conducted in cultured tumor cells to assess the extent of pH reporter uptake. Results are summarized below and in Appendix 5.

Several results were apparent.

i) Signals from tumors were generally very weak whichever reporter molecules were administered i.p or i.v.

ii) Since CF_3-POL did not enter cells, it could be used to measure extra cellular pH only.

iii) Although CF_3-POL gave only a single signal it was relatively broad (Figure 13 below), which may be attributed to heterogeneity of pH in tumors. Since the chemical shift range is relatively small extensive heterogeneity made signal interpretation difficult.

iv) Our prototype molecule, FPOL, was not appropriate as a substitute for CF_3-POL, since the pKa is 8.2, and thus, pHs encountered *in vivo* and at the edge of the titration (chemical shift) range (Fig 13).

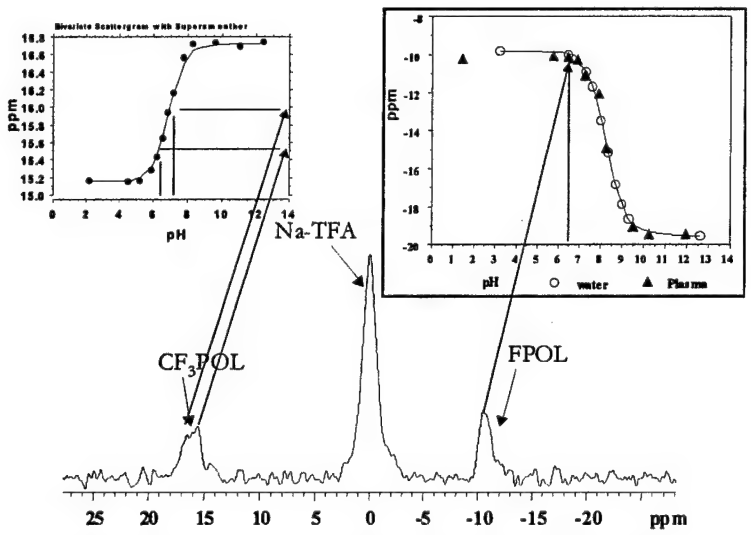


Figure 13 CF_3-POL and FPOL detected in rat breast tumor by ^{19}F NMR

v) Our alternative first generation indicator, FPAM, was not appropriate since it overlapped with ^{19}F NMR signals from the anesthetic gas isoflurane. Hitherto, we had used methoxyflurane, but this has become largely unobtainable and very expensive in the USA. Isoflurane is a better

anesthetic from the point of view of veterinary anesthesia and animal physiology, but signals occur at 5 and 12 ppm coinciding with the range for FPAM (Figure 14 below)

vi) 3APP, the classic ^{31}P NMR indicator developed by Gillies et al [Gillies, 1994 #844] provided only very weak signals in our rat tumors at 4.7 T. It can be used for measurements of extra cellular pH, but requires dual tuned or tunable coils, if it is to be used in conjunction with pharmacokinetics of 5FU. Inevitably, multi resonance coils offer lower signal sensitivity. One might hope to measure transmembrane pH gradient based on 3APP for extra cellular pH and endogenous inorganic phosphate (Pi) for intracellular pH. However, our tumors generally showed minimal Pi.

vii) Our data based on CF_3-POL and FPOL suggest an extracellular pH range of 7.1 to 7.2 in rat breast 13762 tumors. This appears rather high when compared with many other tumors types reported in the literature, but is validated by our own polarographic electrode pH measurements, as shown in the distribution in Figure 14.

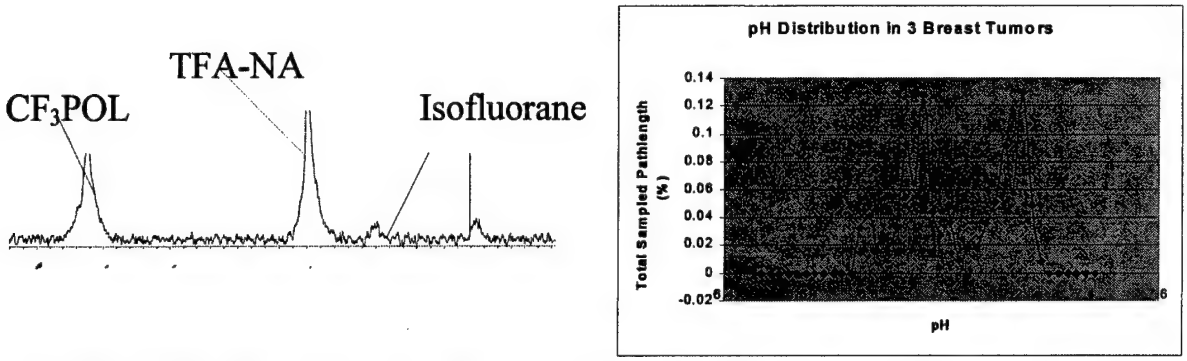


Figure 14 ^{19}F NMR of breast tumor and frequency histogram for polarographic measurements
Task completed

Task 10 Synthesis gram quantities of optimal molecule for *in vivo* use.
Synthesis of 6-FPAM and CF_3-POL was scaled up to facilitate *in vivo* investigations in rats and mice.
Task completed

Phase 3 Relationship of transmembrane pH gradient and 5-FU pharmacokinetics

Task 11 5-FU pharmacokinetics in rat small breast tumors as function of ΔpH.
Although we could not measure the transmembrane pH gradient, we undertook combined studies with the ^{19}F NMR pH indicator and 5FU. On some occasions both signals could be detected. However, 5FU was generally very transient and accompanied by metabolite signals. Higher MR field, such as 7 T could give significantly improved SNR. Representative results are presented.
Task completed, but results not as successful as hoped.

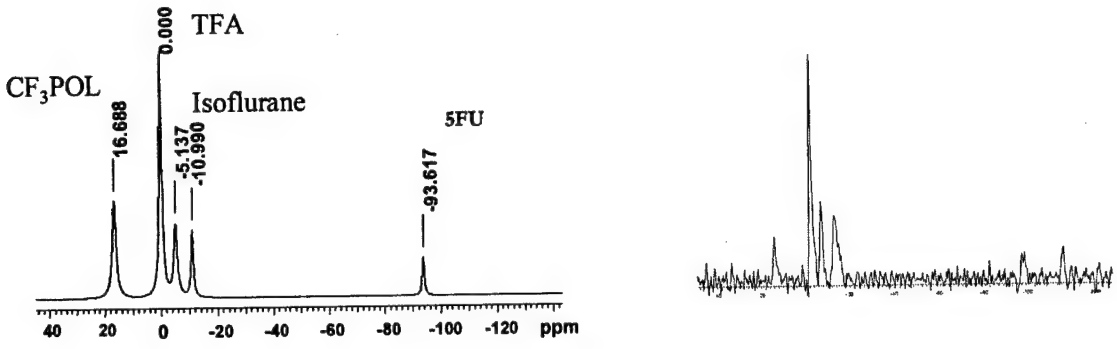


Figure 15 ^{19}F NMR spectrum of CF_3-POL and 5FU in 13762NF breast tumor on anesthetized rat, but with more typical spectrum at right

Task 12 Modulate 5-FU pharmacokinetics in rat small breast tumors: pH gradient manipulation.
We attempted to alter tumor pH using inhaled gas (carbogen) or glucose. Some minor responses were detected, but the SNR of the pH indicators could not efficiently define the response. Representative results are presented.
Task completed, but results not as successful as proposed.

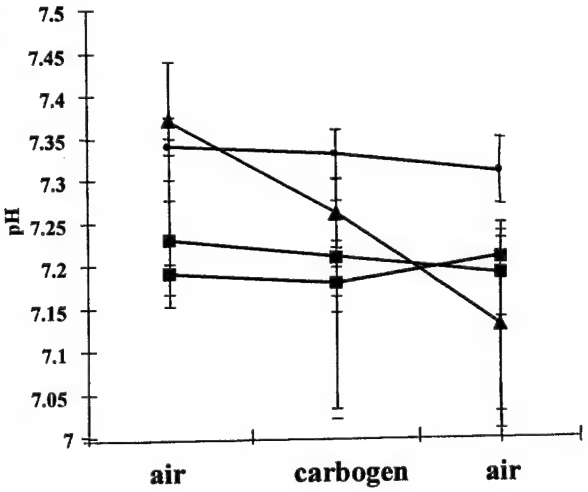


Figure 16 Manipulation of tumor extracellular pH by breathing carbogen in representative breast tumors.

Task 13 Prepare reports and manuscript.
Task completed

Task 14 5-FU pharmacokinetics in rat large breast tumors as function of ΔpH.
As for Task 11, similar problems were encountered in large breast tumors. While the amount of signal was expected to be greater from a larger tumor, the heterogeneity is also more severe, so that signals can be broader. We are attempting to undertake volume selective or chemical shift selective imaging in the hope of achieving narrower lines and local pH. We believe such an approach will be crucial to future application of the pH indicators.
Task completed, but results not as successful as proposed.

Tasks 15- 17 Due to the problems encountered in Tasks 11-14, we could not undertake tasks 15-17. Instead, we continue to try to implement localized spectroscopy in order to achieve better assessment of

spectral lines. We also synthesized several additional candidate pH indicators in Task 3 in an attempt to achieve a useful trans membrane pH indicator. Given that the goal of this project was to measure transmembrane pH gradients in breast tumors, we explored other approaches to stimulate cellular uptake of indicators. As a fallback strategy we tried to use FPOL or FPAM. However, we were surprised to find that these did not enter breast tumor cells. Earlier experiments had shown facile entry into red blood cells and perfused hearts. We proceeded to undertake extensive studies of diverse cell types in culture and finally found some hepatoma cells, which showed uptake of FPAM providing estimations of transmembrane pH gradients, as described in the abstract in Appendix 1 and Figure 17.
In terms of chemical modification, we synthesized derivatives with sugar moieties, which have been reported to facilitate cellular uptake. Results are presented Task 3 and Appendix 5. We continue to evaluate the utility of these molecules.

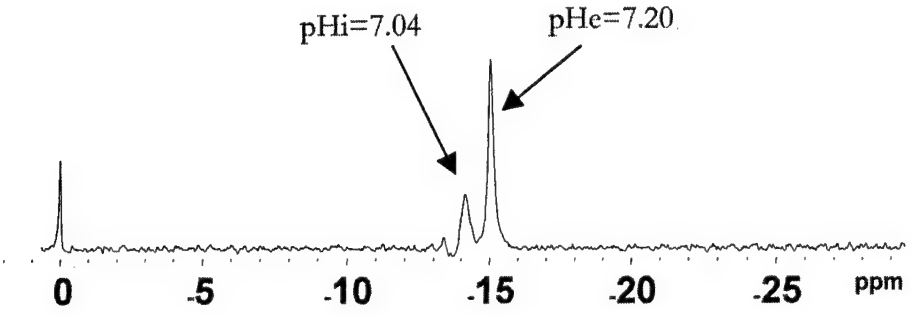


Figure 17 Uptake of FPAM by hepatoma cells (MH3924) transfected to express thymidine kinase

In vivo experiments were also delayed because the 4.7 T MR system was out of commission for several months during 2002. Ultimately, the system has been upgraded to a state of the art Varian INOVA unity system and shows good stability and enhanced sensitivity.

Task 18 Prepare final reports and manuscript.
Task Completed with submission of this report

Key Research accomplishments

- Successful synthesis of the ^{19}F NMR pH indicator CF_3-pyridoxol (primary research molecular target).
- Evaluation of titration curves for CF_3-pyridoxol pH indicator in solution and characterization in whole blood and perfused organs. Verification that CF_3POL does not enter cells and could thus be used to specifically measure extracellular pH, which is considered by some to be more critical than the transmembrane pH gradient.
- Identification of isoflurane signals in ^{19}F NMR spectrum. These can normally be neglected in ^{19}F MRI since the short T2 removes signals from spin echo images. However, in spectroscopy intense signals are found and can potentially interfere.
- Identification and evaluation of fluoronitro phenols as possible alternate ^{19}F NMR pH indicators. These have the potential advantage that they are readily available commercially.
- First demonstration of the entry of 6-fluoropyridoxamine into cancer cells permitting non-invasive measurement of transmembrane pH gradient.

Reportable outcomes

Seven papers describing work supported by this grant were accepted for international conferences as posters or oral presentations including the International Society of Magnetic Resonance in Medicine, the Era of Hope Meeting The Eighth International Conference on Tumor Microenvironment and Its Impact on Cancer Therapies and 38th National Organic Symposium Thee are all included in Appendix 1.

1 "A novel NMR reporter molecule for transmembrane pH gradients: para-fluoro-ortho-nitrophenol". W. Cui, P. Otten, M. Merritt, and **R. P. Mason**, ISMRM 10th Scientific Meeting, Honolulu, Hawai'i, USA 18 - 24 May, 2002

2 "Gene reporter molecules: a novel approach revealing β-galactosidase activity". **R. P. Mason**, P. Otten, Y. Li and K. Koeneman, ISMRM 10th Scientific Meeting, 384, Honolulu, Hawai'i, USA 18 - 24 May 2002

3 "Breast tumor pH: design and evaluation of novel reporter molecules", **R. P. Mason**, P. Otten, W. Cui & J. Yu, Era of Hope, Orlando, P49-10, Sept. 2002.

4 "Transmembrane pH gradient in tumor cells measured by 6-Fluoropyridoxamine, a promising NMR reporter molecule", W. Cui, Z. Ma, S. He, P. Peschke and R. P. Mason, The Eighth International Conference on Tumor Microenvironment and Its Impact on Cancer Therapies, Miami (South Beach), Florida, May, 2003

5 "Novel *in vivo* Gene Reporter Molecule Using Fluorinated Vitamin B_6 as ^{19}F NMR indicator" J. Yu & **R. P. Mason**, Proc. 11th ISMRM, 675, Toronto, May 2003

6 "6-trifluoromethyl pyridoxol, a novel reporter molecule for tumor extracellular pH", J. Yu, P. Otten, W. Cui, **R. P. Mason**, Proc. 11th ISMRM, 623 (2003)

7 "Design, synthesis and evaluation of series compounds based on trifluoromethylated vitamin B6 as potential novel indicators for in vivo and non-invasive detection of tumor cellular pH", J. Yu,

P. Otten, W. Cui, **R. P. Mason**, 38th National Organic Symposium, B2, Bloomington, Indiana, June 2003.

One paper has been accepted for publication, so far, and several more are in preparation.

"A novel NMR approach to assessing gene transfection: 4-fluoro-2-nitrophenyl-β-D-galactopyranoside as a prototype reporter molecule for β-galactosidase", W. Cui, P. Otten, Y. Li, K. S. Koeneman, J. Yu and R. P. Mason, Magn. Reson. Med., accepted (2003)

Results and or training provided by this project were critical in providing preliminary data to successfully win **further grants**:

1 Grantor: National Institutes of Health/NCI P20 CA086354
Title of Project: Southwestern In vivo Cancer Cellular and Molecular Imaging Center (Pre-ICMIC) Principal Investigator: RPM
Total amount of award and dates: $1, 200, 000 5/01-4/04
This is a planning grant for an In vivo Cancer Cellular and Molecular Imaging Center to stimulate greater interaction among imaging scientists, oncologists, and basic scientists.

2 Grantor: NIH/National Center for Research Resources P41 RR02584
Title of Project: Southwestern NMR Center for in vivo Metabolism
Principal Investigator: Craig Malloy
Total amount of award and dates: $5, 526, 214 8/01-7/06
This umbrella grant provides MR infrastructure.

3 Department of Defense Breast Cancer Initiative BC022001
Breast Cancer Gene Therapy: Development of Novel Non-Invasive Magnetic Resonance Assay to Optimize Efficacy
Mason (PI) 05/03-04/06
This grant builds on the observations in the current grant that PFONPG provides a useful NMR signal, which is sensitive to pH and gene activatable from a parental conjugate.

4 Department of Defense Prostate Cancer Initiative PC031075
Broad spectrum chemotherapy: a novel approach using β-galactosidase activated pro-drugs
LI LIU
This grant builds on the observations in the current grant that certain fluoronitrophenols have broad spectrum cytotoxicity and this may be activated by enzyme action

5 Department of Defense (DOD), Department of the Army PC020829
A Novel Approach to Monitoring Prostate Tumor Oxygenation: Proton MRI of the Reporter Molecule Hexamethyldisiloxane
Cui (PI) 12/02 -11/04

Dr. Cui won this grant based on her initial training and experiences in biomedical NMR in the current grant

6 Grantor: DOD Breast Cancer Initiative DAMD17-02-1-0592, BC011290
Title of Project: A BOLD Magnetic Resonance Approach to Breast Tumor Evaluation
(Pre-doctoral fellowship)
Lan Jiang $60,000 5/02-4/05
Based on the research infra structure developed in my group for breast cancer Lan Jiang won this training grant.

Training

Pieter Otten, Ph.D. postdoctoral synthetic organic chemist researcher moved to Chembridge Pharmaceuticals
Jianxin Yu, Ph.D. postdoctoral researcher, now faculty and key investigator on Department of Defense Breast Cancer Initiative BC022001 (Mason, PI) 05/03-04/06
"Breast Cancer Gene Therapy: Development of Novel Non-Invasive Magnetic Resonance Assay to Optimize Efficacy"
Weina Cui, Ph.D. postdoctoral researcher in NMR who has now won Department of Defense (DOD), Department of the Army post doctoral award PC020829 (12/02 -11/04)
"A Novel Approach to Monitoring Prostate Tumor Oxygenation: Proton MRI of the Reporter Molecule Hexamethyldisiloxane"

Individuals receiving salary support during the course of this grant.
Anca Constantinescu
Weina Cui
Eric Hahn
Ralph Mason
Pieter Otten
Jian-Xin Yu

Conclusions

- CF_3 analogues of F-pyridoxine pH indicators can be synthesized and, as predicted, show a sensitivity ~ 1.5 ppm which is much smaller than for Fluoropyridoxal, but comparable to endogenous inorganic phosphate. ^{19}F resonances are sufficiently narrow to allow potential NMR discrimination of pH.
- Synthesis of the fluoropyridoxol is complex and requires highly skilled organic chemist. We thus evaluated several "off the shelf" commercial agents as possible alternate pH indicators. While some had interesting pH sensitivity, several were highly toxic or exhibited poor water solubility. These results justify the synthetic undertaking proposed in this grant.
- ~~CF_3-POL does not enter cells. While this prevents the goal of the current grant to measure~~ transmembrane pH gradients, it ensures that measurements reliably reveal extra cellular pH (pHe). This can be important since agents which have the ability to enter cells, but still show only a single resonance raise the question of whether there is truly no trans membrane gradient or whether the agent happened to fail to enter. This may be very important, since intra cellular pH can often be determined based on endogenous inorganic phosphate by ^{31}P NMR. pHe has been largely elusive and new indicators are needed. We will continue to evaluate the utility of this agent.
- For the first time we have observed 6-fluoropyridoxamine enter tumor cells. Studies would likely be more successful at higher magnetic field. Localized spectra could overcome the broadening associated with tumor heterogeneity.

Appendix 1

1 1 "A novel NMR reporter molecule for transmembrane pH gradients: para-fluoro-ortho-nitrophenol". W. Cui, P. Otten, M. Merritt, and **R. P. Mason**, ISMRM 10th Scientific Meeting, Honolulu, Hawai'i, USA 18 - 24 May, 2002

2 "Gene reporter molecules: a novel approach revealing β-galactosidase activity". **R. P. Mason**, P. Otten, Y. Li and K. Koeneman, ISMRM 10th Scientific Meeting, 384, Honolulu, Hawai'i, USA 18 - 24 May 2002

3 "Breast tumor pH: design and evaluation of novel reporter molecules", **R. P. Mason**, P. Otten, W. Cui & J. Yu, Era of Hope, Orlando, P49-10, Sept. 2002.

4 "Transmembrane pH gradient in tumor cells measured by 6-Fluoropyridoxamine, a promising NMR reporter molecule", W. Cui, Z. Ma, S. He, P. Peschke and R. P. Mason, The Eighth International Conference on Tumor Microenvironment and Its Impact on Cancer Therapies, Miami (South Beach), Florida, May, 2003

5 "Novel *in vivo* Gene Reporter Molecule Using Fluorinated Vitamin B_6 as ^{19}F NMR indicator" J. Yu & **R. P. Mason**, Proc. 11th ISMRM, 674, Toronto, May 2003

6 "6-trifluoromethyl pyridoxol, a novel reporter molecule for tumor extracellular pH", J. Yu, P. Otten, W. Cui, **R. P. Mason**, Proc. 11th ISMRM, 623 (2003)

7 "Design, synthesis and evaluation of series compounds based on trifluoromethylated vitamin B6 as potential novel indicators for in vivo and non-invasive detection of tumor cellular pH", J. Yu, P. Otten, W. Cui, **R. P. Mason**, 38th National Organic Symposium, B2, Bloomington, Indiana, June 2003.

Manuscript in the press

"A novel NMR approach to assessing gene transfection: 4-fluoro-2-nitrophenyl-β-D-galactopyranoside as a prototype reporter molecule for β-galactosidase", W. Cui, P. Otten, Y. Li, K. S. Koeneman, J. Yu and R. P. Mason, Magn. Reson. Med., accepted (2003)

A novel NMR reporter molecule for transmembrane pH gradients: para-fluoro-ortho-nitrophenol

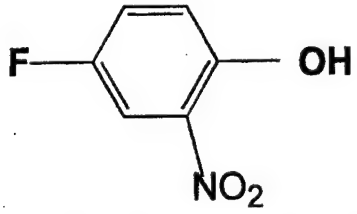
W. Cui, P. Otten, M. Merritt, and R. P. Mason,
Department of Radiology, UT Southwestern Medical Center, Dallas, TX

pH regulation is critical in many disease states; in particular it is thought to impact the efficacy of various cytotoxic therapies for cancer. Even more significant may be transmembrane pH, since this influences the distribution of weakly acidic or basic drugs. We have now identified para-fluoro-ortho-nitrophenol as a promising new sensor for measuring transmembrane pH gradients. We present titration curves together with initial measurements in solution and whole blood showing the veracity of this new approach.

Introduction

Many diseases generate perturbations in tissue physiology revealed by deficits in perfusion, oxygenation and pH. NMR provides a non-invasive means to monitor these parameters and dynamic changes in response to interventions. Previously, derivatives of vitamin B6 (6-fluoropyridoxol; FPOL) have been proposed for measuring transmembrane pH in blood, perfused heart and tumors (1), but synthetic preparation is laborious. Para-fluoro-ortho-nitrophenol (PFONP) is readily available commercially and presents structural characteristics compatible with a useful pH sensor molecule. We have now evaluated PFNP and present data showing its efficacy as an NMR reporter molecule.

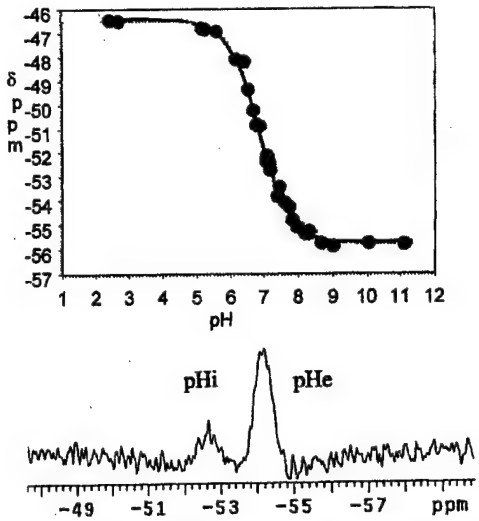
Materials and methods



PFONP was obtained from Aldrich. NMR characteristics and titration curves were assessed at 383 and 596 MHz at 25 °C or 37 °C in saline and plasma. Samples were added to fresh whole heparinized rabbit blood together with sodium trifluoroacetate (TFA) as a chemical shift reference and 6-fluoropyridoxol as an alternate pH indicator.

Results

PFONP has a single ^{19}F NMR signal in water. There are small H-F couplings, but these are generally not resolved in biological samples. The ^{19}F signal is highly sensitive to pH exhibiting a range of 9.3 ppm between acid and base as shown in the titration curve. The pKa = 6.85 and the Henderson Hasselbalch coefficients are δ_{acid} –46.44 ppm, δ_{base} –55.75 ppm with respect to TFA = 0 ppm. Even at neutral pH there is no excessive line broadening, though the water solubility is substantially lower under acidic conditions.



Partial 564 MHz ^{19}F NMR spectrum from whole rabbit blood containing 6 mg PFONP acquired in 2 mins.

The titration curves were found to be identical in saline or plasma. Upon addition to whole blood, two signals attributed to intra- and extra-cellular compartments are rapidly observed, *e.g.*, at –52.68 and –54.21 ppm corresponding to pHi = 7.16 and pHe = 7.55, as shown in spectrum above. Co-addition of 6-fluoropyridoxol indicated pHi = 7.29 and pHe 7.55 and an electrode showed pHe = 7.66. Signal assignment was confirmed by centrifuging the blood to separate cells and plasma components. T1 in whole blood was in the range 0.9 to 1.5 s.

Discussion

While many pH indicators have been proposed for NMR, the greatest hurdle is generally achieving cellular penetration in order to provide the trans membrane pH gradient based on a single reporter molecule observed in a homo nuclear experiment. PFONP shows pH sensitivity competitive with the best molecules reported hitherto and its ready commercial availability is attractive. Since the phenol is a weak acid, it must be added judiciously to biological samples so as not to perturb the intrinsic pH. Toxicity is in the range 400 mg/kg in mice. We are initiating studies to explore the range of applications for this novel indicator.

Reference

1 Mason, R. P., *Curr. Med. Chem.* **6**, 481, 1999

Research supported by grants from Department of Defense (DAMD17-99-1-9381) and NCI P20 CA86354.

Gene reporter molecules: a novel approach revealing β-galactosidase activity

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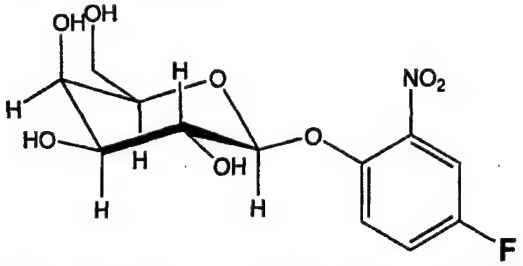
Gene therapy is generating increasing interest. However, a major issue is the success of targeting the gene to tissue of interest, the distribution of the gene, its activity and longevity of action. To this end various strategies have been proposed to detect gene expression. We present a novel concept: ^{19}F NMR detection of β-galactosidase activity based on the chemical shift change accompanying cleavage of the enzyme substrate para- fluoro- ortho- nitro- phenyl β-D-galactopyranoside. Here, we report the MR characteristics of this novel reporter molecule together with examples of its application *in vitro* and *in vivo*.

Introduction

The activity of most therapeutic genes is not directly detectable. A powerful tool is the incorporation of a tandem reporter gene, which reveals activity of genes of interest. Historically, the most popular reporter gene has been lac-z, which generates β-galactosidase (β-gal). Numerous biochemical assays are available to detect β-gal activity, but they have been limited to histology or *in vitro* assays. Recently, a ^{1}H MR contrast reporter molecule was presented (1), but this bridged cyclic paramagnetic agent requires complex synthesis, fails to penetrate cells and is a poor substrate for the enzyme. We reasoned that introduction of a fluorine atom into the traditional biochemical substrate ortho-nitro-phenyl galactopyranoside (ONPG), could provide a novel enzyme activity sensor (*viz.* gene reporter) with minimal perturbation to a well proven substrate.

Materials and methods



Para- fluoro- ortho- nitro- phenyl β-D-galactopyranoside (PFONPG) was synthesized using the methods of Yoon *et al.* (2). Following purification, the molecule was assessed at 9.4 and 14.1 T in various solutions, whole rabbit blood and cultured prostate tumor cells.

Results

PFONPG was isolated in good yields and found to have a single ^{19}F NMR signal at –42.66 ppm (with respect to sodium trifluoroacetate). The molecule was stable in water and whole rabbit blood for a period of hours.

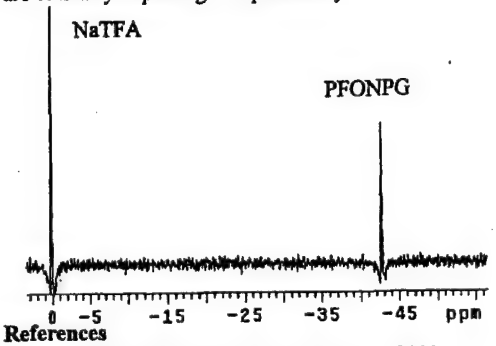
Addition of β-gal (aspergillus oryzae, Sigma) led to rapid cleavage liberating the aglycone, which appeared as a new peak ~ 5 ppm upfield, accompanied by color development. Incubation of PFONPG with cultured human prostate cancer cells (PC3 and C4-2) showed no enzyme activity. However, when these cells were infected overnight with adenovirus carrying the β-gal gene driven by CMV promoter, gene activity was revealed by liberation of the aglycone. Most significantly, the rate and extent of activity were in line with expected levels of gene expression.

Discussion

Introduction of the fluorine atom into the classical biochemical reagent ONPG provides a novel NMR reporter molecule to detect β-gal activity. Given the well known promiscuity of β-gal, diverse substrates could be developed, but maintaining close structural similarity to existing indicators may be most appropriate. The ^{19}F resonance of PFONPG does show small F-H coupling and ^{1}H decoupling could improve the signal to noise ratio. However, the coupling is generally not seen in biological samples due to inherent line broadening.

^{19}F NMR has the great advantage that there is essentially no background signal in tissue. Moreover, cleavage of the PFONPG provides definitive indication of enzyme activity revealed by the change in chemical shift. Nonetheless, studies may be limited to spectroscopy by the concentrations achievable *in vivo*.

The approach presented here, adds to the choice of methods for investigating gene transfection. Furthermore, the aglycone PFONP chosen here shows intrinsic pH sensitivity raising the intriguing possibility of determining local pH at the site of β-gal activity. We are currently exploring this possibility.



References

1. Louie, *et al. Nature Biotechnol.* **18**, 321, 2000
2. Yoon, *et al. Bull. Korean Chem. Soc.* **17**, 599, 1996

Supported by NCI P20 CA86354 and DAMD17-99-1-9381

BREAST TUMOR PH: DESIGN AND EVALUATION OF NOVEL REPORTER MOLECULES

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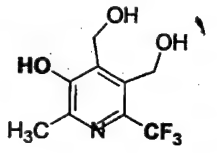
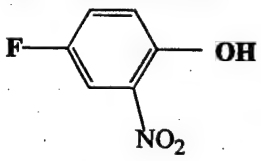
Many factors impact the efficacy of cytotoxic therapy for breast cancer. Local acidity (pH), and in particular, cellular transmembrane pH gradients can influence the distribution of therapeutic drugs. Historically, no satisfactory methods existed to measure transmembrane pH gradients *in vivo*. We have investigated new approaches using magnetic resonance reporter molecules and have identified two novel classes of agent.

6-(trifluoromethyl)pyridoxine (CF_3-POL; a vitamin B6 analogue) exhibits a chemical shift of 1.6 ppm between acid and base conditions and has a pKa = 6.7 making it ideal for investigations of breast cancer. Initial tests show that it provides reliable pH measurements in whole blood. The CF_3 group provides a three-fold gain in signal to noise over our previous prototype agents [*Curr. Med. Chem.* 6, 481 (1999)]. Unfortunately, CF_3-POL does not penetrate cancer cells and is restricted to providing estimates of extra cellular pH only. This can itself be valuable, but we are also exploring methods of stimulating cellular penetration and surveying various cell types for uptake characteristics.



CF_3-POL

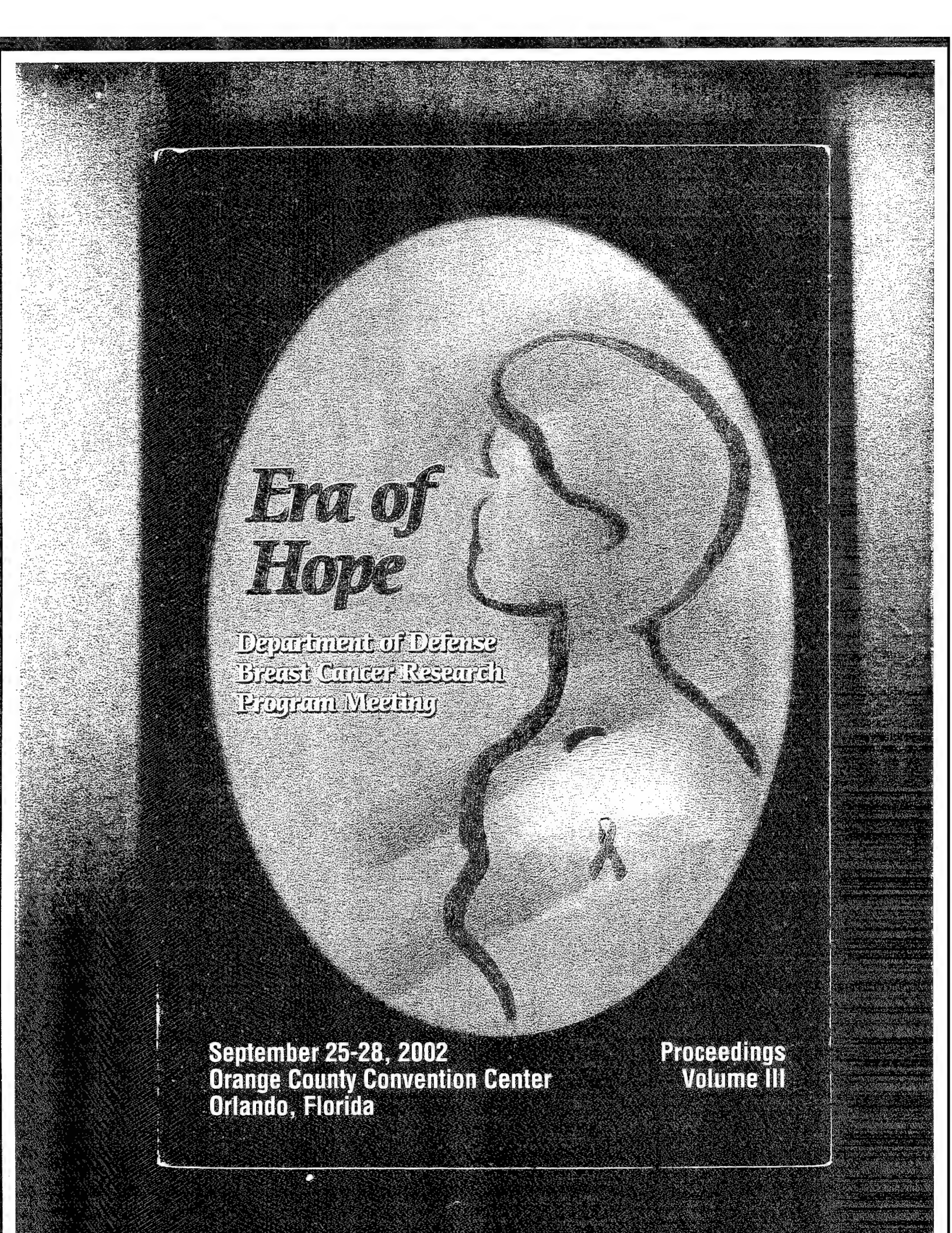


PFONP

Para-fluoro-ortho-nitrophenol (PFONP) represents a novel class of pH sensor molecule. The single ^{19}F NMR signal exhibits a chemical shift range of 9.3 ppm between acid and base and pKa = 6.85. Upon addition to whole blood, two signals attributed to intra- and extra-cellular compartments were rapidly observed, *e.g.*, –52.68 and –54.21 ppm corresponding to pHi = 7.16 and pHe = 7.55. We are initiating studies of pH in breast tumors with this novel indicator. PFONP offers additional intriguing opportunities: it is a fluorinated analogue of the aglycone in ONPG (orthonitrophenol) the classical test reagent for β-galactosidase activity in histology. We have synthesized the corresponding glycoconjugate (PFONPG) and it is indeed a very sensitive reporter for enzyme activity.

The new reporter molecules will be valuable for assessing the relevance of tumor pH. Such measurement may facilitate improved therapeutic outcome based on the characteristics of a specific tumor. PFONPG opens the possibility of monitoring gene expression *in vivo* and interrogating local pH at sites of gene activity, which may impact the field of gene therapy.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9381 supported this work.


Era of Hope
Department of Defense
Breast Cancer Research
Program Meeting
September 25-28, 2002
Orange County Convention Center
Orlando, Florida
Proceedings
Volume III

Transmembrane pH gradients in tumors:
observations using ^{19}F NMR of promising new reporter molecules

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pH plays an important role in tumor proliferation, metabolic control, and response to therapy. In particular, it has been shown that the tumor cell transmembrane gradient influences the biodistribution and efficacy of many chemotherapeutic agents. We have been developing novel pH indicators and report progress in the development of useful ^{19}F NMR reporter molecules. We had shown that 6-fluoropyridoxol (FPOL, a vitamin B6 analog) could be used to measure transmembrane pH gradients in blood and perfused hearts (*Curr. Med. Chem.* 6, 481, 1999). Recently, we applied FPOL to *in vivo* studies of rat breast tumors. Baseline measurements showed only one signal suggesting either lack of cellular penetration, lack of pH gradient or inability to differentiate the signals due to the basic pKa = 8.2 of FPOL. Nonetheless, we obtained intriguing results associated with altering inhaled gas in tumor bearing rats. Switching from air to carbogen breathing had no apparent effect on pH of the rat tumor, but return to air produced significant transient acidification. Control experiments with cultured cells suggest lack of cell penetration. We have now examined new indicators to investigate transmembrane pH gradients. 6-fluoropyridoxamine (FPAM) is a close analog of FPOL which requires sophisticated organic synthesis but the substance has a large chemical shift response to pH ($\Delta\delta_{acid\text{-}base}$ = 10.1 ppm) and a pKa = 7.05. Like FPOL, FPAM shows two signals in whole blood and perfused hearts.

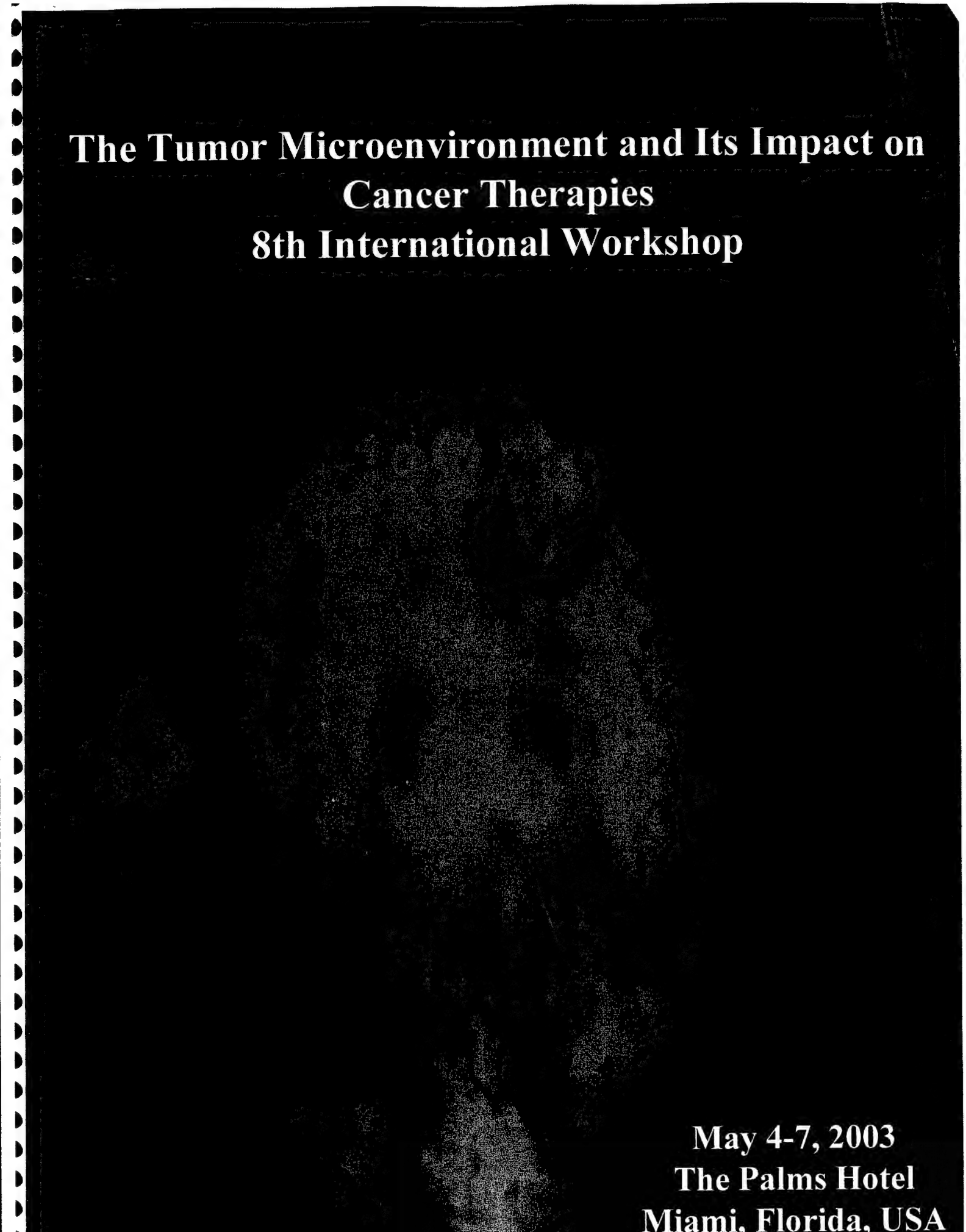
Wild-type and transfected cells (Morris Hepatoma, Chinese Hamster Ovary) were cultured in RPMI 1640 medium plus 10% fetal bovine serum, 100 IU/ml penicillin and 100μg/ml streptomycin at 37°C under 5% CO_2. The cells were trypsinized and suspended in TRIS-EDTA (TE; pH 8.2) buffer with a cell concentration around 10^7/ml. Experiments were performed at 9.4 T with a capillary of sodium trifluoroacetate as an external chemical shift reference. Mesurements.

Upon addition of FPAM to suspensions of Hepatoma MH3924A, MH-Tk or MH-DmdNK1 cells, and Chinese Hamster Ovary Cells in TE buffer, two signals attributed to intra and extra cellular compartments were rapidly observed, *e.g.* -15.81 ppm and –17.84 ppm corresponding to pHi = 7.34 and pHe = 7.86 in MH3924A cells, –15.67 ppm and –17.80 ppm corresponding to pHi = 7.31 and pHe = 7.84 in MH-DmdNK1 cells, -14.16 ppm and –15.04 ppm corresponding to pHi = 7.04 and pHe = 7.20 in TK cells, and -16.32 ppm and –18.11 ppm corresponding to pHi = 7.45 and pHe = 7.97 in CHO cells. The extra cellular pH was confirmed by pH electrode and analysis of supernatant and corresponded with the basic TE buffer.

Unlike FPOL, the pH indicator FPAM shows two signals in cultures of several types of mammalian cells. Greatest uptake was observed in the hepatoma cells transfected to express thymidine kinase (TK), but other cells also showed some intra cellular signal. ^{19}F NMR pH indicators could be particularly useful since they may be observed simultaneously with various drugs such as 5-fluorouracil and many novel physiological and gene reporter molecules. We are now initiating studies using FPAM to investigate measurements of transmembrane pH gradients *in vivo*.

This work was supported by DOD BrCa Initiative DAMD 17-99-1-9381, NIH RO1 CA79515, and NCI Pre-ICMIC P20 CA86354.

The Tumor Microenvironment and Its Impact on Cancer Therapies
8th International Workshop



May 4-7, 2003
The Palms Hotel
Miami, Florida, USA

Novel in vivo Gene Reporter Molecule Using Fluorinated Vitamin B6 as 19F NMR indicator

J. Yu[1], R. P. Mason[1]
[1]UT SWMED CTR. @ Dallas, Dallas, Texas, United States

Synopsis

A novel *in vivo* gene reporter molecule **GFPOL** was designed, synthesized and evaluated. The ^{19}F NMR spectra indicate that β-galactosidase was able to efficiently activate **GFPOL** releasing the pH indicator 6-fluoropyridoxol.

Introduction

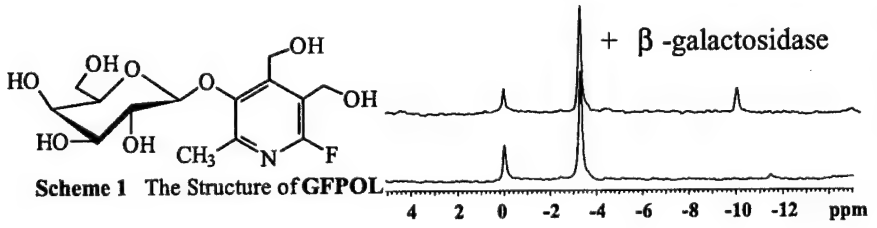
The *E. coli lacZ* gene, encoding the enzyme β-galactosidase, has been widely applied to the study of problems in cell and molecular biology, oncology, and the recently emerging fields of genomics and proteomics. To detect the presence of the *β*-galactosidase using the well established chromogenic or fluorogenic substrates, the sample must be fixed and appropriately stained yielding rather crude estimates, and limited to histology or *in vitro* assays. We have proposed a novel concept: ^{19}F NMR detection of β-galactosidase activity based on the ^{19}F chemical shift change accompanying cleavage of enzyme substrates. The first generation reporter PFONPG (*p*-fluoro-*o*-nitrophenol galactopyranoside) was an effective substrate for β-galactosidase entering cells and exhibiting a suitable ^{19}F NMR chemical shift respsone to enzyme cleavage [1]. This represented an exciting substrate since the aglycone used was pH sensitive presenting the concept of gene activated local pH measurement. Unfortunately, PFONPG is highly toxic. We have now explored alternative conjugates and demonstrate a similar capability using a well-known pH indicator 6-fluoropyridoxol (6-**FPOL**) [2]. We now present a novel gene reporter molecule **GFPOL** (Scheme 1) and its primary properties for *in vivo* and non-invasive gene expression detection.

Materials and Methods

GFPOL was first stereo- and regioselectively synthesized and characterized in our lab. NMR spectra were recorded on a Varian MERCURY 400 spectrometer (376MHz for ^{19}F) with Tris-Cl buffer as solvents, a dilute solution of sodium trifluoroacetate (NaTFA) in a capillary as external standard. Enzymic reactions were conducted at 37°C in 30 mM Tris-Cl buffer (pH 8.0).

Results

GFPOL has good aqueous solubility, and shows a single ^{19}F NMR signal. It is stable in water and whole rabbit blood for a period of hours. The hydrolytic reactions were monitored by ^{19}F chemical shift changes from NMR spectra. The ^{19}F NMR spectra indicate that β-galactosidase was able to hydrolyze **GFPOL** effectively providing a change of 8.0 ppm with hydrolysis (Fig. 1 and 2). Furthermore, the aglycone **FPOL** has been demonstrated as a pH indicator raising the fascinating possibility of determining local pH at the site of β-galactosidase activity.



Scheme 1 The Structure of **GFPOL**

Fig. 1

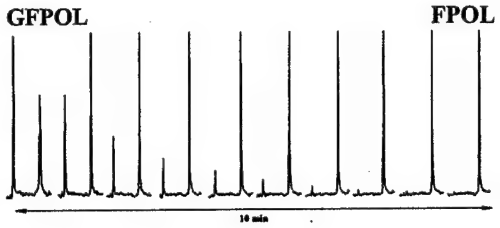


Fig. 2

Discussion

The successful synthesis of **GFPOL** and demonstration of sensitivity to β-galactosidase further validates this approach to probing gene activity. ^{19}F NMR can be highly effective, since there is essentially no background tissue signal. Here, we have demonstrated the ability to add a known pH indicator to a gene reporter construct providing a combined reporter molecule. We are currently exploring its application *in vivo*.

Acknowledgements Supported by DOD Initiative BC980020 DAMD17-99-1-9381 and NCI Pre-ICMIC P20 CA086354.

References [1] R. P. Mason, P. Otten, Y. Li, K. Koeneman, *ISMRM*, p71, 2002. [2] R. P. Mason, *Curr. Med. Chem.*, **6**, 481, 1999.

6-trifluoromethyl pyridoxol, a novel reporter molecule for tumor extracellular pH

W. cui[1], P. otten[1], J. yu[1], V. kodibagkar[1], R. P. Mason[1]
[1]UT Southwestern Medical center at Dallas, Dallas, TX, United States

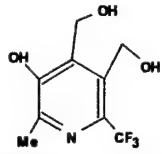
Synopsis

6-trifluoromethyl pyridoxol, a derivative of vitamin B6, was evaluated as an extracellular pH indicator. ^{19}F NMR spectroscopy shows that the chemical shift difference of this molecule between acid and base is 1.65ppm, and pKa is 6.82 ideal for physiological condition. We present titration curves at different temperatures, together with measurements in solution, whole blood, perfused heart, rat breast and prostate tumors, showing the efficacy of 6-trifluoromethyl pyridoxol as a novel pH indicator.

Introduction

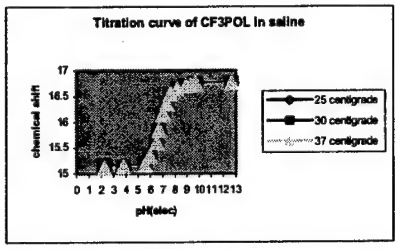
pH plays an important role in tumor proliferation, angiogenesis, metabolic control and various cytotoxic therapies for cancer. Many methods for pH measurement have been developed. Among them, NMR is noninvasive, non-destructive and can penetrate deep tissues. Previously, one NMR reporter molecule, the derivative of vitamin B6, 6-fluoropyridoxol(FPOL), has been proposed for measuring transmembrane pH in blood, perfused heart and tumors[1]. But the pKa of FPOL is 8.2, which is not ideal for measurements under normal physiological conditions. To enhance the signal to noise (trifluormethyl as opposed to F-atom), 6-trifloromethyl pyridoxol(CF_3POL), was designed and synthesized. We have now evaluated CF_3POL and present data showing its efficacy as an NMR reporter molecule.

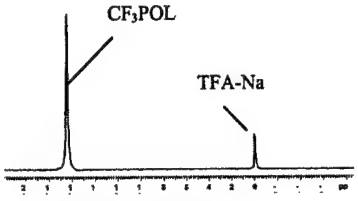
Materials and methods



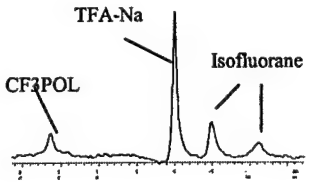
CF_3POL was synthesized by our lab. NMR titration curves in saline were assessed at 376 MHz at 25 °C, 30 °C and 37°C. Fresh whole heparinized rabbit blood was used and sodium trifluoroacetate is a chemical shift reference for all experiments. Langendorff retrograde perfusion was performed with recycled phosphate-free, modified Kres-Henseleit buffer oxygenated with carbogen at 37 °C under a pressure of 100cm H_2O. For in vivo experiments, 320mg/kg CF_3POL in saline was injected intraperitoneally into anesthetized Copenhagen rat with pedicle prostate tumor (2.4*3.1*1.8) or Fisher 344 rat with pedicle breast tumor (1.8*1.0*2.0), and ^{19}F NMR spectroscopy was performed at 4.7T.

Results

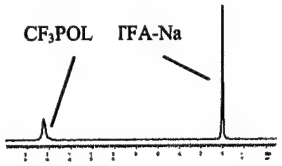




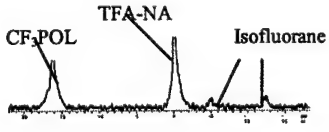
CF_3POL in whole blood



CF_3POL in prostate tumor



CF_3POLin perfused heart



CF_3POL in breast tumor

CF_3POL has a single narrow signal throughout the pH range in saline or blood. As expected, the sensitivity to pH is less than FPOL since the electron distribution for pH sensitive para-OH bond pass through additional C-C bond. The chemical shift difference between acid and base is 1.64 ppm, the pKa of CF_3POL is 6.82 and is insensitive to temperature. In perfused heart, ^{19}F signal of CF_3POL occurs at 16.41ppm corresponding to pH=7.39, while electrode showed pHe=7.28. Phosphorous spectrum showed δPi=4.88 corresponding to intracellular pH=7.04. CF_3POL is detected in tumor 30min after the injection, showing at 16.28ppm corresponding to pH=7.20 in prostate tumor, and 16.23ppm corresponding to pH=7.14 in breast tumor. Tumors showed additional upfield signals from anesthetic isofluorane.

Discussion

CF_3POL has an ideal pKa=6.82 for physiological measurement, shows pH sensitivity in saline, whole blood, perfused heart, and possesses the ability to enter rat tumor. CF_3POL is hydrophilic and only one-third dosage is needed to be used relatively to FPOL. Although, CF_3POL only provides extracellular pH, intracellular pH can be assessed by inorganic phosphorous signal from ^{31}P NMR spectroscopy. We believe 6-trifluoromethyl pyridoxol has distinct promise as a new indicator for measuring extra cellular pH in vivo.

Acknowledgement

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Reference

1. Mason, RP. . Curr. Med. Chem. 6, 481, 1999

DESIGN, SYNTHESIS AND EVALUATION OF SERIES COMPOUNDS BASED ON TRIFLUOROMETHYLATED VITAMIN B_6 AS POTENTIAL NOVEL INDICATORS FOR IN VIVO AND NON-INVASIVE DETECTION OF TUMOR CELLULAR PH

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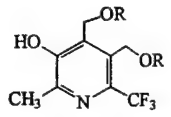
pH plays a significant role in cellular regulation and strongly influences drug uptake. Thus, the measurement of pH in tumors promises insight into developmental processes and prognostic information regarding therapeutic outcome. We have previously described the vitamin B6 analog 6-fluoropyridoxol (FPOL) [*Curr. Med. Chem.*, **6**, 481, 1999] as a sensitive ^{19}F NMR pH indicator exhibiting a chemical shift range of 10 ppm. It readily crosses cell membranes and we demonstrated application to measure transmembrane pH gradients in whole blood and perfused hearts. With the goal of improving the signal-to-noise ratio, we have now evaluated a series of second generation reporters, using a CF_3-group instead of a single F-atom. The chemical shift difference between the acid and its conjugate base is 1.64 ppm compared to 10 ppm for FPOL. The rationale for the lower sensitivity is that the reporting F-atoms are not in direct π communication with the pH sensitive *para*-OH bond. The pK_a of CF_3POL is ideal at 6.82 and is insensitive to temperature. We will present synthetic strategies, molecular and spectral characteristics, and applications to cells, perfused organs, and tumors *in vivo*.



R = H, $(CH_2)_2OH$, $(CH_2)_2NH_2$, β or α-D-Glcp, $(CH_2)_2O$-D-Glcp, $(CH_2)_2NHCSNH$-D-Glcp

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Manuscript in Press

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A novel NMR approach to assessing gene transfection: 4-fluoro-2-nitrophenyl-β-D-galactopyranoside as a prototype reporter molecule for β-galactosidase*

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Running title: NMR detection of β-galactosidase activity

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Keywords: β-galactosidase, gene reporter molecule, lacZ, ^{19}F NMR, nitrophenyl-β-D-galactopyranoside, prostate cancer

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Abstract

Gene therapy holds great promise for the treatment of diverse diseases. However, widespread implementation is hindered by difficulties in assessing the success of transfection in terms of spatial extent, gene expression, and longevity of expression. The development of non-invasive reporter techniques based on appropriate molecules and imaging modalities may help to assay gene expression. 4-fluoro-2-nitrophenyl-β-D-galactopyranoside (PFONPG) is a novel prototype NMR sensitive molecule, which is highly responsive to the action of β-galactosidase (β-gal), the product of the lacZ gene. The molecule is stable in solution and with respect to wild type cells, but the enzyme causes very rapid liberation of the aglycone, accompanied by color formation and a ^{19}F NMR chemical shift of 5 to 10 ppm, depending on pH. Since the product is pH sensitive, this opens the possibility of direct pH determinations at the site of enzyme activity. Molecular and ^{19}F NMR characteristics of PFONPG in solution, blood, and prostate tumor cells are presented. This prototype molecule introduces a novel approach for assaying gene activity *in vivo*.

Introduction:

Gene therapy holds great promise for treatment of diseases including cancer, cystic fibrosis, and immuno deficiency. However, a major hurdle to widespread successful implementation is the need to verify successful transfection, in particular, the spatial extent of expression in the target tissue, together with assays of the longevity of expression. An image based assay would greatly facilitate optimal gene therapy vector dosing, in a precise temporal and spatial manner. Numerous pre-clinical studies reveal initial promise for treatment of solid tumors. However, useful reporter molecules could accelerate the effective transition to human clinical trials.

Many promising methods are being developed to image (assay non-invasively) tissue gene expression often by including a reporter gene in tandem with the therapeutic gene (1-4). A critical criterion is that the reporter gene not be normally present or expressed in the cells of interest. One of the earliest examples of gene transfection was the introduction of creatine kinase into the liver of mice and subsequent detection of phosphocreatine (PCr) by ^{31}P NMR (5). This had the advantage of a benign product and natural substrates, but would be inappropriate in most tissues, where PCr is normally present at high levels. Perhaps the most popular reporter genes today are associated with optical imaging, since this is a cheap modality and highly sensitive results are rapidly available. Thus, bioluminescent imaging (BLI) of luciferase (1) and fluorescent imaging of green fluorescent protein (GFP and longer wavelength variants (6)) are popular. These techniques are very useful in superficial tissues and have extensive applications in mice, but application to larger bodies is limited by depth of light penetration.

Several nuclear medicine approaches have been demonstrated exploiting thymidine kinase with a variety of substrates including iodo- and fluoro-nucleosides, such as FIAU and gancyclovir, and various radionuclide labels including 123-, 124-, 125-I, and ^{18}F (3, 7). An alternative approach uses the sodium iodine symporter (hNIS), which works well with both

iodide and pertechnetate substrates (2). For cancer, thymidine kinase has the advantage that the gene serves not only as a reporter, but gene products can themselves have therapeutic value.

The lac operon was the first gene expression system to be well characterized, some forty years ago by Jacob and Monod. One component, lacZ, which produces β-galactosidase, has been the primary choice of reporter gene to verify effective transfection in biochemistry, and many reporter molecules are available for biological and histological analysis. Diverse agents are commercially available with specific characteristics, such as developed color, thermal stability, and cellular retention (*e.g.*, X-gal, ONPG (o-nitrophenylgalactoside), and S-Galacton-star) (8-10). However, β-galactosidase had been largely neglected for *in vivo* work, until the elegant studies of Meade *et al.* (11). The galactose bridged cyclic gadolinium contrast agent ((1-(2-(galactopyranosyloxy)propyl)-4,7,10-tris(carboxymethyl)-1,4,7,10–tetraazacyclo dodecane) gadolinium(III) (EgadMe) shows considerable change in water relaxivity upon exposure to β-galactosidase. While the molecule is a poor substrate for the enzyme (of the order of 500 times less efficient than the colorimetric biochemical agent ONPG) and does not penetrate cells, it facilitated effective investigation of cell lineage following direct intra-cellular microinjections (11). These studies prompted us to consider other NMR active analogs, and it appeared that introduction of a fluorine atom into the popular colorimetric biochemical indicator ONPG could produce a strong candidate molecule. We now report successful synthesis of the reporter molecule, together with NMR characterization and examples of activity in solution and in cell culture.

Materials and Methods:

4-fluoro-2-nitrophenyl-β-D-galactopyranoside (PFONPG, Fig. 1) was prepared from a reaction of acetobromo-α-D-galactose and the potassium salt of 4-fluoro-2-nitrophenol (PFONP) followed by deprotection with triethylamine using methods closely analogous to those reported

by Yoon *et al* (12). Details of the chemical synthesis will be published in due course (manuscript in preparation).

^{19}F NMR experiments were performed at 564 MHz using a Varian INOVA Unity spectrometer and a capillary of sodium trifluoroacetate served as an external chemical shift reference standard (δ 0 ppm). Spectra were obtained of both PFONPG and the aglycone PFONP in saline, buffers, heparinized whole rabbit blood and prostate cancer cells. Titration curves of 4-fluoro-2-nitrophenol were measured at 25 °C and 37 °C in saline and aliquots of HCl (0.2N) and NaOH (0.25N) were added to alter pH, which was independently measured in the NMR tube using a pH electrode.

For single component kinetic enzyme experiments PFONPG (5 mg) was dissolved in buffer-I (0.6 ml, pH = 4.5, prepared with 10 mM sodium hydrogen phosphate and 5 mM citric acid). A solution of β-galactosidase (G5160 from *Aspergillus oryzae*, Aldrich; 10 μl of a solution of 19 mg (152 units) in 2 ml buffer-I) was added and NMR data were acquired immediately at 30 °C. Each spectrum was acquired in 36 s and the kinetic curve assessed over 11 min. A similar experiment was performed using β-gal isolated from *Escherichia coli* (G6008; 250-600 units/mg) in buffer-II at 37 °C. Buffer-II was prepared with HEPES (2 mM) and hydrogen phosphate (7 mM) and providing the higher pH optimal for this enzyme (pH = 7.3).

To test substrate efficacy, a substrate competition experiment was undertaken. NMR experiments were performed with various PFONPG concentrations (28.8 μmol to 262 μmol) added to a solution of β-gal (G5160). In a second series 2-nitrophenyl-β-D-galactopyranoside (ONPG, 65 μmol) was added simultaneously to each sample.

For cell studies, $5x10^6$ human prostate tumor cells PC-3 (American Type Culture Collection, Manassas, VA) and LNCAP C4-2 (UroCor, Oklahoma city, OK) were grown on 150 mm culture dishes in T-medium (Invitrogen) with 5% fetal bovine serum at 37 °C with 5% CO_2. Control (wild type) cells and transfected cells (infected by replication defective adenovirus harboring lacZ

gene under the control of the CMV (cytomegalo virus) or BSP (bone sialo protein) promoter at 10 or 100 MOI (multiplicity of infection) with transfection time of 24 hr or 48 hr were harvested. Cells were harvested by trypsinizing for 2 minutes and neutralizing with medium. The cell pellet was obtained by gentle centrifugation, washed twice with phosphate buffered saline (PBS) and resuspended in 1 ml PBS. PFONPG (2 mg) was added to suspension of 10^7/ml cells and ^{19}F NMR spectra were acquired after various incubation times at 30 or 37 °C.

Results

PFONPG is hydrophilic and readily dissolves in saline or whole blood giving a single narrow ^{19}F NMR signal at δ -42.75 ppm with respect to $\delta_{Na\text{-}TFA}$ 0 ppm (Fig. 2). This signal is essentially invariant in the range pH 1 to 11 with a change of < 0.05 ppm. The signal appeared stable in solution or whole rabbit blood for a period of 2 days. Addition of β-galactosidase caused rapid cleavage releasing the aglycone, PFONP, which appeared at δ -46.49 ppm for β-gal G5160 at 30 °C and at δ -51.07 ppm for β-gal G6008 at 37 °C (Fig. 3) and was accompanied by development of yellow color. Addition of β-gal (G5160) to PFONPG showed rapid exponential loss of the substrate accompanied by appearance of the aglycone over a period of 10 mins. as shown by the curves in figure 4. Similar activity was seen with G6008, though the change was less rapid under comparable conditions. The substrate competition kinetics showed that ONPG acts as a competitive inhibitor of β-gal with respect to PFONPG: the Michaelis constant increased from 91 μmol to 200 μmol, but Vmax remained unchanged (Fig. 5).

When PFONPG was added to wild type prostate cancer cells (PC-3 or LNCAP C4-2) there were no spectral changes after incubation for 3 hrs at 37 °C. When PC-3 cells were transfected with a first generation adenovirus vector encoding the β-gal gene driven by the CMV promoter for 24 hrs, 12.7% PFONPG was converted to PFONP after incubation for 3 hr at 37 °C (Table 1). When PC-3 cells or LNCAP C4-2 cells were transfected with the CMV system, but for 48 hrs, 74% PFONPG was converted by PC-3 cells and 100% by LNCAP C4-2 cells after incubation for

15 min at room temperature. CMV may be considered a universal promoter and we also tested the β-gal gene under control of the specific BSP promoter. In this case the substrate cleavage was lower with only 5% conversion after incubation with PFONPG for 3 h at 37 °C for cells with 10 fold MOI viral transfection and 14% for 100 MOI.

The ^{19}F NMR signal of the aglycone (PFONP) is very sensitive to pH exhibiting a range of 9.3 ppm. The titration curves (Fig. 6) were identical at 25 °C and 37 °C and gave the Henderson Hasselbalch coefficients pKa =6.85, δ_{acid} -46.44 ppm, δ_{base} -55.73 ppm.

Discussion

We have demonstrated the potential utility of a novel class of gene reporter molecule, fluoro-phenyl-galactopyranosides, specifically, 4-fluoro-2-nitrophenyl-β-D-galactopyranoside (PFONPG) as an effective substrate for β-galactosidase. This molecule is an excellent substrate for the enzyme and acts competitively with traditional biochemical indicators. It provides a single ^{19}F NMR signal with a narrow linewidth and good stability in solution. It is apparently stable in normal wild type cells and whole blood, but exposure to the enzyme or cells transfected to express β- galactosidase causes rapid cleavage in line with anticipated levels of transfection.

Upon cleavage of the glycosidic bond a chemical shift difference > 3.6 ppm is observed. However, the chemical shift of the product may have a range of about 9 ppm, since the released aglycone is pH sensitive and the pKa is in the physiological range. Significantly, there is no overlap between the chemical shift of the substrate and the product. This presents the interesting possibility of selective determination of pH at the site of enzyme activity. Indeed, we have demonstrated that if the aglycone (PFONP) is added to a suspension of red blood cells, two signals are rapidly observed representing the intra and extra cellular pH (13). However, PFONP is somewhat toxic and causes lysis of less robust cells such as cultured tumor cells. Thus, PFONPG may be regarded as an interesting prototype molecule primarily representative of a new approach to NMR gene reporter molecules for use in association with β-galactosidase.

Given the well known promiscuity of β-galactosidase many commercial colorimetric molecular substrates are available for this enzyme. We believe that other NMR sensitive aglycone analogs may readily be introduced in place of the PFONP and can display preferable characteristics. In particular, analogs may be selected to be less toxic, be selectively trapped in cells or be selected to have particular activities, as specific reporters or drugs. Indeed, preliminary investigations show the feasibility of conjugating the pH reporter 6-fluoropyridoxol (14) to galactose, generating an effective substrate for β-gal (15).

While the toxicity of PFONP appears to severely limit the application of PFONPG as a gene reporter molecule, it does open the intriguing vista of a broad spectrum gene activated chemotherapeutic. Indeed, PFONP is clearly analogous to the classic biochemical uncoupler dinitrophenol. While specific traditional chemotherapeutic drugs may be subject to multi drug resistance and become ineffective, we believe that nitrophenols could exert local cytotoxic efficacy on many tissues. Gene activated drug therapy, often termed GDEPT (gene directed enzyme prodrug therapy) (16), has been demonstrated by others using the cytosine deaminase (CD) gene. Specifically, CD activates the minimally toxic 5-fluorocytosine (5-FC) to the highly toxic 5-fluorouracil (5-FU). This is being widely exploited in gene therapy trials, in the hope of mitigating the toxicity threshold associated with systemic 5-FU delivery (16, 17). The conversion of 5-FC to 5-FU causes a chemical shift ~1.5 ppm, hence, revealing gene activity, which has been demonstrated in a number of systems *in vivo* (16, 18). It is also interesting to note that some of the major metabolic products of 5-FU exhibit chemical shift sensitivity to pH and these could provide an indication of local tissue pH (19).

NMR reporters have the great advantage over radioactive substrates of a long shelf life and ease of handling. Functional paramagnetic agents are attractive, since they interact with large numbers of water molecules generating amplification, as shown for various diverse: "smart" contrast agents (20). However, the prototype proton NMR substrate for β-gal (EgadMe) fails to

enter cells and is a poor substrate for the enzyme. Introduction of a fluorine atom minimally perturbs the structure and reactivity of the traditional efficient biochemical substrate (ONPG) and yields a new approach. Given that there is essentially no ^{19}F NMR background signal in tissues, fluorinated reporter molecules may be assessed by changes in chemical shift and the detection ability is subject to the signal-to-noise ratio, as opposed to contrast-to-noise. The chemical shift difference between substrate and aglycone product reveals unambiguous detection of enzyme activity and this approach has been demonstrated for the cytosine deaminase reporter gene system previously (16, 18). Spatial resolution will require chemical shift imaging, rather than merely selective excitation, since the PFONP can have a wide pH dependent chemical shift range. Detectability could be enhanced by introduction of a trifluoromethyl (CF_3) reporter group, as opposed to the single F-atom. However, a CF_3 group will likely perturb the water solubility to a greater extent and the chemical shift response is expected to be considerably smaller due to transmission of the electron density redistribution through an additional carbon-carbon bond.

PFONPG is readily synthesized using facile organic chemical methods. PFONPG is water soluble and appears to enter cells with ease. However, the product aglycone is not trapped and in some cases causes cell lysis. Thus, while cleavage provides clear evidence of β-galactosidase activity, it would be difficult to localize this information to specific tissues *in vivo.* This may limit the ultimate application of this prototype molecule for widespread studies.

Acknowledgments

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Figure legends

Figure 1.

The structure of 4-fluoro-2-nitrophenyl-β-D-galactopyranoside (PFONPG).

Figure 2

^{19}F NMR spectra of PFONPG (2 mg≡8 μmol, 10 mM) in saline (Left) and fresh heparinized whole rabbit blood (right). ^{19}F NMR spectra were obtained in 36 s and apodized with a 10 Hz exponential line broadening prior to Fourier transformation. Sodium trifluoroacetate was used a chemical shift reference (δ 0 ppm).

Figure 3

PFONPG (-42.75 ppm) was rapidly cleaved by β-gal G5160 (pH = 4.5, upper spectrum) and G6008 (initial pH = 7.3, but acidified to 6.8 during reaction, lower spectrum) releasing the aglycone PFONP. Since the product has a pH sensitive chemical shift, PFONP occurs at different positions for each buffer.

Figure 4

Kinetic curves showing cleavage of PFONPG to PFONP by β-gal G5160. Upper trace shows spectral time course and amplitudes are plotted below.

Figure 5

Lineweaver-Bourke plot showing kinetics of β-gal activity on PFONPG and competitive inhibition by addition of ONPG (o).

Figure 6

Titration curves of 4-fluoro-2-nitrophenol (PFONP) in saline (♦), and plasma at 30 °C (□), and 37 °C (Δ).

Table 1

Cell line	PC3	PC3	PC3	C4(2)
Promoter	BSP	CMV	CMV	CMV
Transfection time	24 hr	24 hr	48 hr	48 hr
Incubation temperature	37 °C	37 °C	room	room
Incubation time	3 hr	3 hr	15 min	15 min
Cleaved portion	5.3%	12.7%	74%	100%

Figure 1

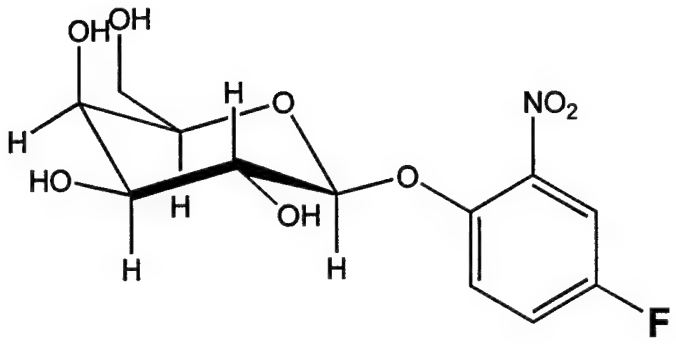


Figure 2

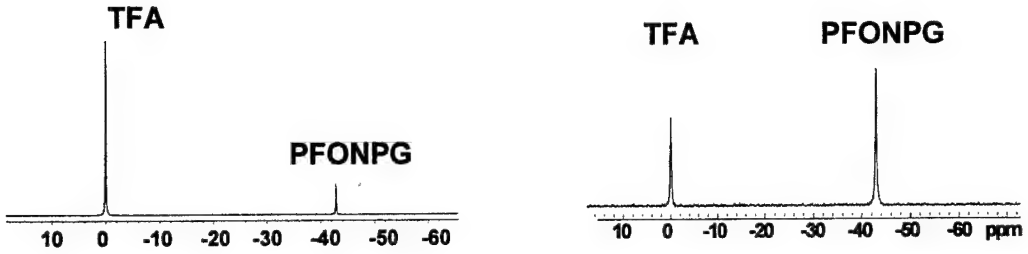

TFA
PFONPG
10 0 -10 -20 -30 -40 -50 -60
TFA
PFONPG
10 0 -10 -20 -30 -40 -50 -60 ppm

Figure 3

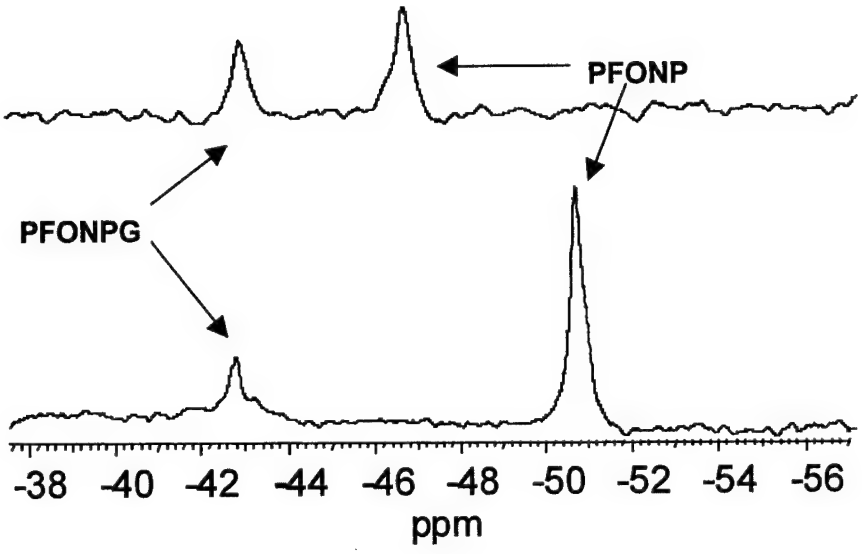
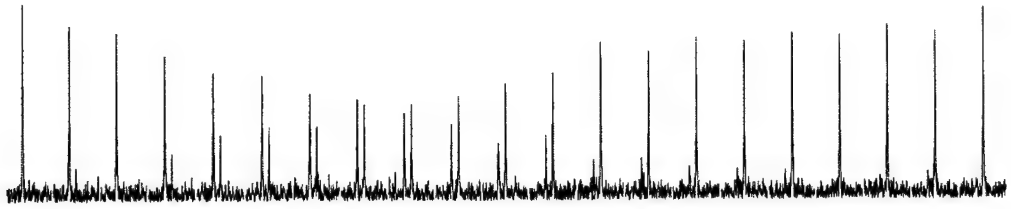


Figure 4



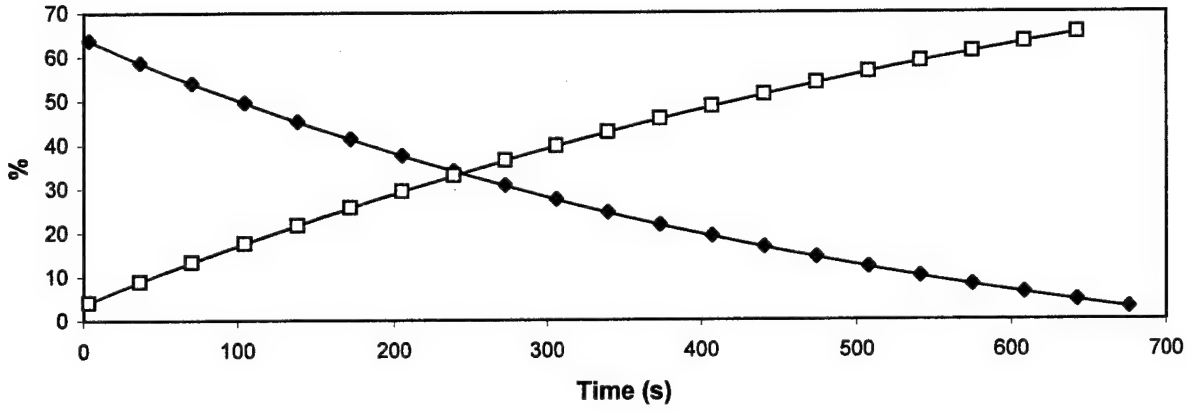
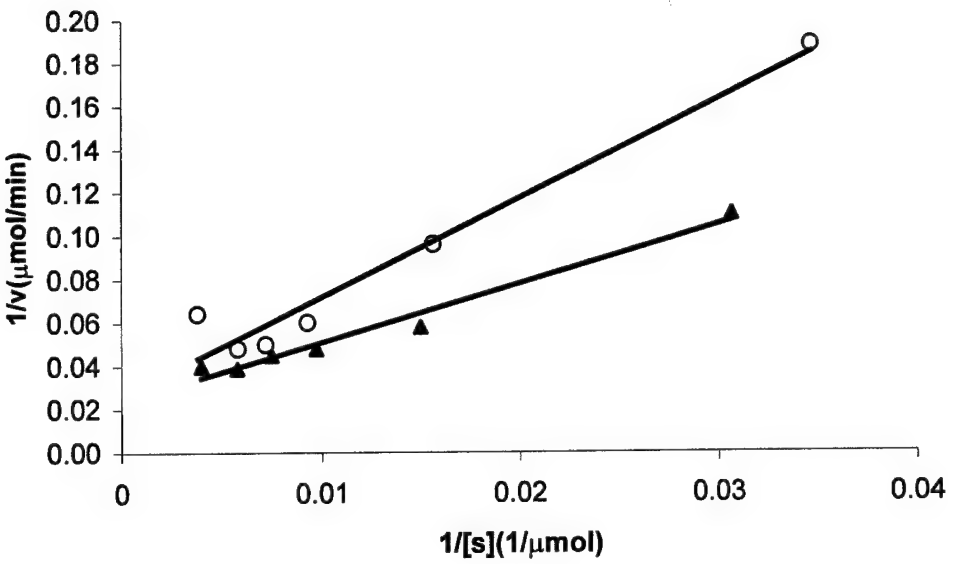


Figure 5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0.18
0.16
0.14
0.12
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0.08
0.06
0.04
0.02
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0.01
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0.03
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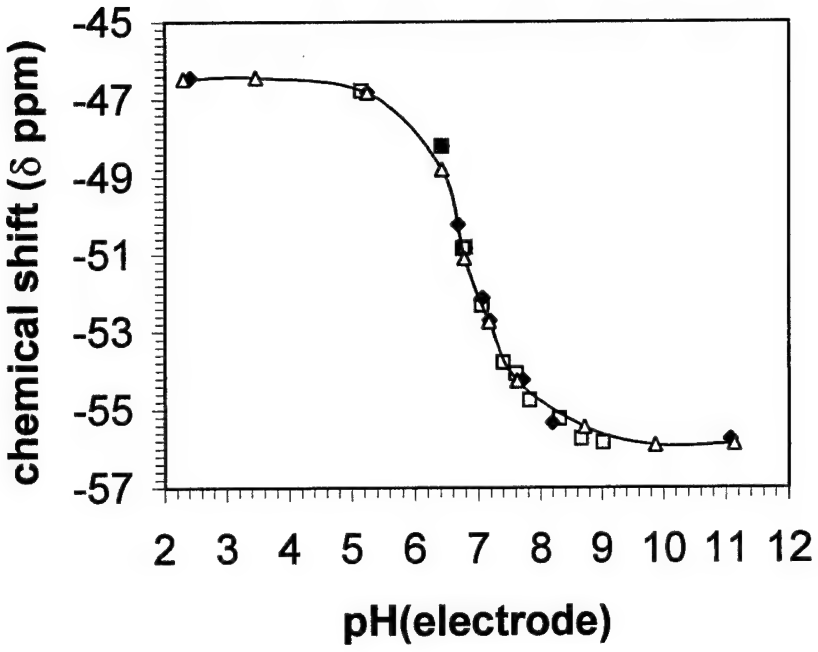
1/[s](1/μmol)

Figure 6


chemical shift (δ ppm)
-45
-47
-49
-51
-53
-55
-57
2 3 4 5 6 7 8 9 10 11 12
pH(electrode)

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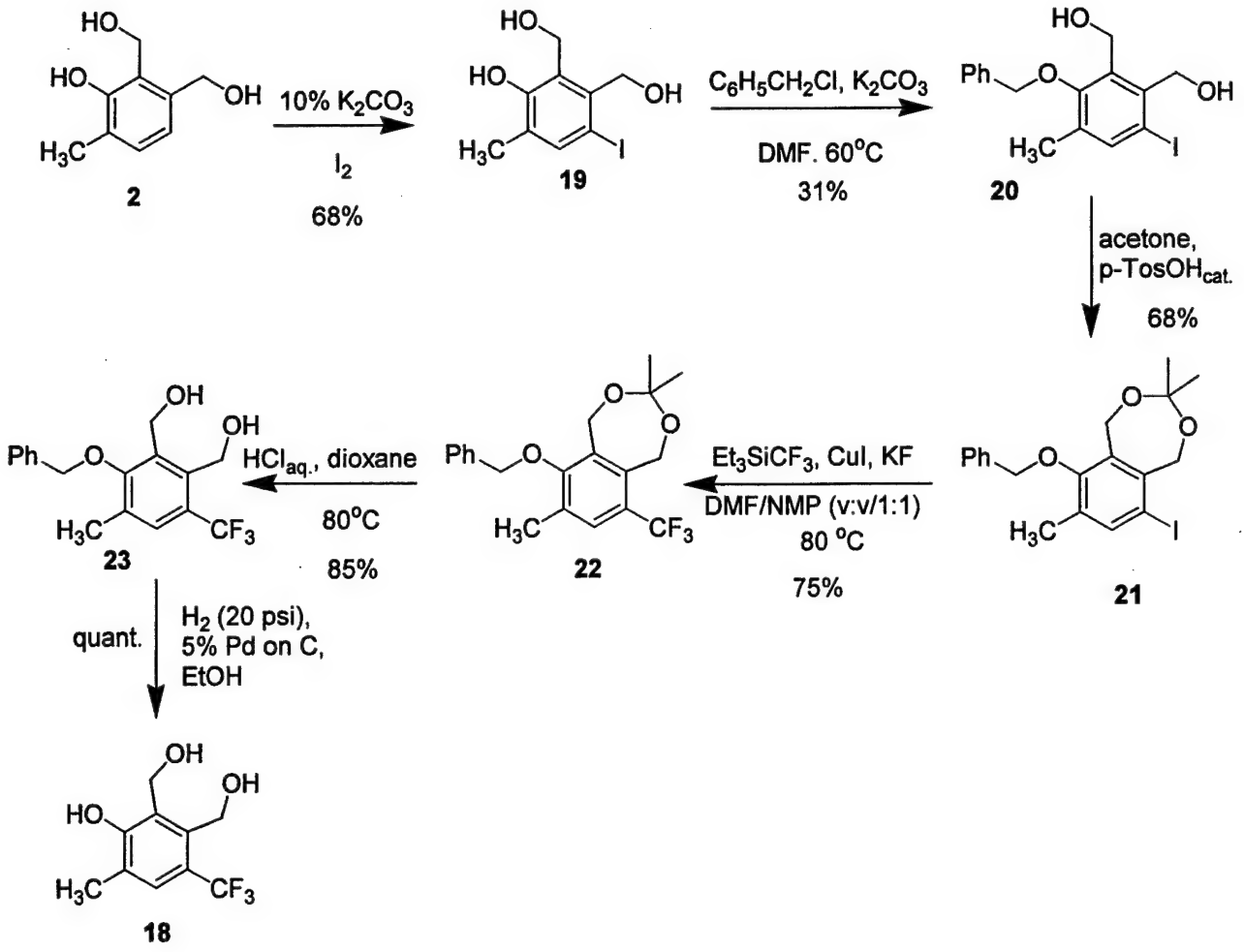
Appendix 2

Development of enhanced pH reporter molecule: trifluoromethyl pyridoxol 6-CF_3POL

Draft manuscript for submission to Bioorganic Medicinal Chemistry letters

The introduction of a trifluoromethyl group at an aromatic ring can be achieved by a substitution reaction on an aryl iodide with trifluoromethyl copper ("$CuCF_3$") [7]. Urata [8] described a convenient method for the *in situ* generation of $CuCF_3$ by a metathesis reaction of commercially available Et_3SiCF_3 with CuI in the presence of an aryl iodide, which serves as the substrate in the trifluoromethylation reaction. For the synthesis of 6-(trifluoromethyl) pyridoxine (**18**), we required access to 6-iodopyridoxine (**19**) and protection of its alcohol groups. The three-step halogenation of pyridoxine (**2**)-via 6-aminopyridoxine (**4**), followed by a Sandmeyer reaction - has been very successful for the synthesis of our ^{19}F NMR pH indicator 6-fluoropyridoxine (**1**) [1, 2, 9, 10]. We decided, however, to attempt the direct iodination of pyridoxine (**2**) using a procedure previously described for the iodination of 3-hydroxypyridine in aqueous base [11]. The reaction of pyridoxine (**2**) with iodine was carried out in 10% K_2CO_3 generating the pyridoxine anion, which is the actual reactive species. Acidification of the reaction mixture resulted in the precipitation of 6-iodopyridoxine. **19** could be obtained pure in a yield of 68% by filtration. Attempts to protect all three hydroxy groups of **19** at once as their respective benzyl ethers (NaH, benzyl chloride, DMF) failed and led to unidentifiable product mixtures. Instead, a two step approach was used. Firstly, the more acidic phenolic hydroxy group was protected as its benzyl ether by using milder basic reaction conditions: K_2CO_3 in warm DMF in the presence of benzyl chloride, which afforded benzyl ether **20** in a modest 31% yield. Next, the two remaining hydroxy groups were protected together in the form of acetone acetal **21**. Acetal **21** was obtained in 68% yield by the reaction of **20** in acetone in the presence of a catalytic amount of *p*-toluenesulfonic acid followed by chromatographic work-up.

We were gratified to see that Urata's trifluoromethylation methodology for phenyl iodides could be extended to a more sterically hindered iodopyridine ring such as our protected iodopyridoxine **21**. Thus, reaction of **21** in a mixture of DMF and NMP (v/v:1/1) in the presence of CuI, Et_3SiCF_3 (b.p. 41 °C), and KF in a sealed tube at 80 °C led to complete conversion of **21** and gave protected 6-(trifluoromethyl)pyridoxine **22** in a yield of 75% after purification by flash chromatography. Subsequent removal of the acetal protection of the aliphatic hydroxy groups was carried out in a mixture of dioxane and 1M HCl at 80 °C. Benzylated (trifluoromethyl)pyridoxine **23** precipitated upon removal of the dioxane from the reaction mixture and was isolated in a yield of 85 % after filtration. The synthesis of the desired 6-(trifluoromethyl)pyridoxine (**18**) was finalized by deprotection of the phenolic hydroxy group by hydrogenolysis of the benzylether **23,** which proceeded in near quantitative yield.



Titrations

The ^{19}F NMR signal of 6-CF_3POL in water shows one sharp line. Using CF_3COONa as a reference, the chemical shift moves downfield from 15.16 ppm (at pH = 2.18) to 16.74 ppm (at pH = 11.08) upon increasing pH. Thus, the chemical shift sensitivity of 6-CF_3POL is a factor 6 less than 6-FPOL. This observation can be readily explained by the fact that in 6-CF_3POL the three reporting fluorine atoms are not in direct communication through resonance with the sensor: the phenolic OH group. The pK_a of 6-CF_3POL was determined at 6.7 compared to 8.2 for 6-FPOL. Apparently, the stronger electron-withdrawing nature of the CF_3 group causes this marked increase in acidity, which makes 6-CF_3POL a better probe for physiological measurements in more acidic tumor tissue. No significant line broadening of the ^{19}F resonance was observed at or around the pK_a value of 6-CF_3POL. In our opinion, this means that despite the reduced chemical shift range, 6-CF_3POL will be useful as a sensitive NMR pH indicator.

In fresh rabbit blood, at 22 °C, 6-CF_3POL shows only one resonance after incubation at 37 °C for 4 hours (2 mg of CF_3POL in 0.5 ml of blood). This observation clearly indicated that no significant amounts, if any at all, of 6-CF_3POL accumulated in the available red blood cells to allow the detection

of an intracellular signal. Raising the NMR probe temperature to 37 °C did not result in the appearance of an additional, intracellular, signal. This is in sharp contrast to our observations for 6-FPOL and its amino derivative 6-FPAM (pK_a = 7.05) which both report an intracellular ^{19}F NMR signal within minutes after mixing with fresh blood. In this particular blood sample, CF_3POL displayed a resonance at 16.49 ppm, which corresponds with an extracellular pH of 7.47 after applying the Henderson-Hasselbach equation. The pH of the sample was determined at 7.39 by a pH electrode.

Methods

6-Iodopyridoxine ( ). To a suspension of 4.25 g (25 mmol) pyridoxine in 75 ml water was added 6.9 g (47 mmol) K_2CO_3 resulting in complete dissolution of all solid material. Subsequently, 6.3 g (25 mmol) iodine was added in one portion followed by stirring for 1 h at room temperature. To the dark brown reaction mixture, 400 mg Na_2SO_3 was added resulting in a yellow clear solution. The reaction mixture was subsequently quenched with concentrated HCl to a pH of 3 and the precipitate was isolated by filtration over a Büchner filter and dried *in vacuo* over sodium hydroxide to give 4.98 g (68 %) of a yellow powder. ^1H NMR (dmso-d_6) δ 2.30 (s, 3H), 4.55 (d, J = 6 Hz, 2H), 4.79 (s, 2H), 5.07 (t, J = 6 Hz, 1H), 5.8 (br, 1H), 9.5 (br, 1H).

6-Iodo-3-*O*-benzylpyridoxine ( ). To a solution of 9 g 6-iodopyridoxine (31 mmol) in 30 ml dry DMF under argon were added 18 g K_2CO_3 (123 mmol) and 12 ml benzylchloride (104 mmol). The reaction mixture was stirred at 60 °C overnight, allowed to cool to room temperature, and filtered over a Büchner filter. The filtrate was poured into 200 ml water and extracted three times with 200 ml EtOAc. The combined organic layers were dried on $MgSO_4$, filtered, and evaporation of the solvent *in vacuo* gave a dark solid, which was triturated with 50 ml diethyl ether. Filtration over a glass filter and drying *in vacuo* over sodium hydroxide gave 4.2 g (35 %) of a pink solid. ^1H NMR (dmso-d_6) δ 2.42 (s, 3H), 4.68 (s, 2H), 4.70 (s, 2H), 4.94 (s, 2H), 7.40-7.54 (m, 5H).

6-Iodo-3-*O*-benzyl-α^4,α^5-*O*-isopropylidenepyridoxine ( ). To a solution of 4.2 g (11 mmol) 6-iodo-3-*O*-benzylpyridoxine ( ) in 60 ml acetone was added 1.2 g *p*-toluenesulfonic acid. The reaction mixture was stirred at room temperature for two days after which another 50 ml of acetone and 1 g of *p*-toluenesulfonic acid were added, followed by an additional stirring for 16h. The reaction mixture was neutralized by the addition of 50 ml water and 3 g K_2CO_3. The acetone was removed *in vacuo* and the remaining aqueous layer was extracted three times with 150 ml EtOAc. The combined organic layers were dried on $MgSO_4$, filtered, and evaporation of the solvent *in vacuo* gave a dark oil which was further purified by flash chromatography (eluent: 10% EtOAc in hexane) to give 3.2 g (68 %) of a white solid. ^1H NMR ($CDCl_3$) δ 1.45 (s, 6H), 2.46 (s, 3H), 4.77 (s, 2H), 4.78 (s, 2H), 4.79 (s, 2H), 7.36-7.42 (m, 5H).

3-*O*-benzyl-α^4,α^5-*O*-isopropylidene-6-(trifluoromethyl)pyridoxine ( ). To a solution of 760 mg (1.8 mmol) 6-Iodo-3-*O*-benzyl-α^4,α^5-*O*-isopropylidenepyridoxine ( ) in a mixture of 2 ml DMF and 2 ml NMP in a 15 ml glass pressure tube were added 510 mg (2.7 mmol) CuI and 125 mg (2 mmol) KF. The well-stirred solution was kept under an argon flow for 30 min followed by the addition of 500 µl Et_3SiCF_3 (2.7 mmol) and the tube was sealed. The mixture was stirred at 80 °C for 16 h and allowed to cool to room temperature. The clear, dark brown reaction mixture was poured into 100 ml water and the resulting white suspension was extracted three times with 200 ml diethyl ether. The combined organic

layers were washed three times with 100 ml water, dried on $MgSO_4$, filtered and evaporation of the solvent *in vacuo* gave a dark oil which was purified by flash chromatography (eluent: 10% EtOAc in hexane) to give 496 mg (75 %) of a colorless viscous oil. ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 6H), 2.48 (s, 3H), 4.78 (s, 2H), 4.79 (s, 2H), 4.80 (s, 2H), 7.40-7.50 (m, 5H).

3-*O*-benzyl-6-(trifluoromethyl)pyridoxine ( ). To a solution of 1.5 g (4.0 mmol) 3-*O*-benzyl-α^4,α^5-*O*-isopropylidene-6-(trifluoromethyl)pyridoxine ( ) in 10 ml 1,4-dioxane was added 5 ml 1 M HCl. The turbid mixture was heated to 80°C, eventually became clear, and stirring was continued for 4 h at this temperature. TLC (eluent: 25% EtOAc in hexane) indicated that all starting material had been consumed. After the reaction mixture was allowed to cool to room temperature, all volatile materials were removed *in vacuo* which resulted in the formation of a white precipitate. The crude product was evaporated to dryness, suspended in 10 ml water and filtered. The residue was dried *in vacuo* on NaOH to give 1.1 g (85 %) of a white powder. ^{1}H NMR (dmso-d_6) δ 2.49 (s, 3H), 4.72 (s, 4H), 5.00 (s, 2H), 7.40-7.45 (m, 3H), 7.52-7.54 (m, 2H).

6-(trifluoromethyl)pyridoxine (6-CF3POL,). To a suspension of 1.0 g (3.1 mmol) of 3-*O*-benzyl-6-(trifluoromethyl)pyridoxine ( ) in 100 ml ethanol was added 500 mg of 5% Pd on C. The mixture was hydrogenolyzed for 16h under 25 psi after which TLC (eluent: 50% EtOAc in hexane) indicated complete consumption of the starting material and the formation of one single product. The reaction mixture was filtered over Celite and the solvent was evaporated *in vacuo* to give 731 mg (99 %) of an off-white solid. ^{1}H NMR (dmso-d_6) δ 2.37 (s, 3H), 4.55 (d, J = 0.9 Hz, 2H), 4.84 (s, 2H).

Acknowledgments

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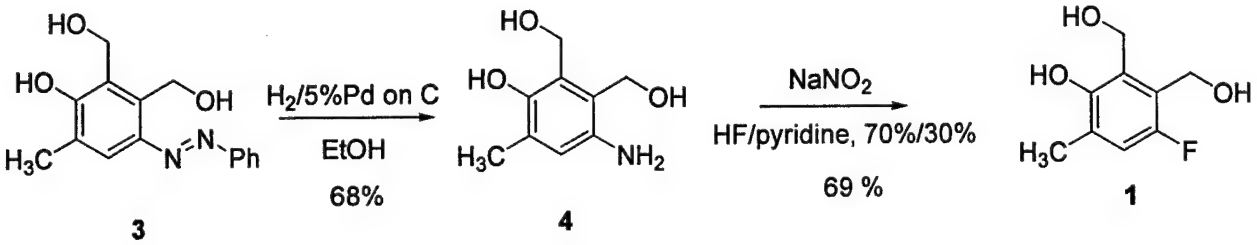
Appendix 3

Draft manuscript for submission to Bioorganic Medicinal Chemistry Letters

Development of novel pH indicator incorporating internal chemical shift reference.

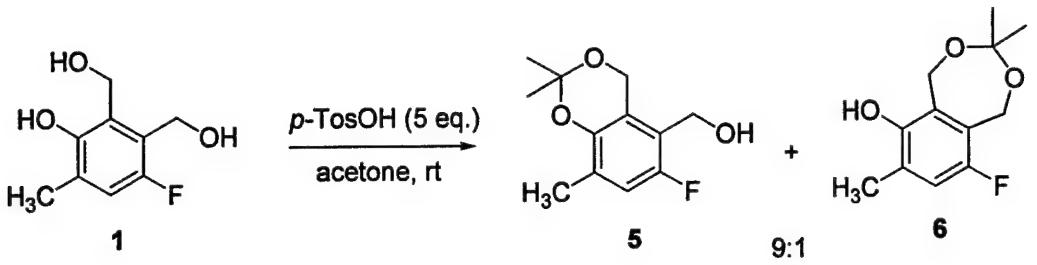
Synthesis

Our first task at hand was to improve the earlier reported synthesis of 6-fluoropyridoxine (6-FPOL, **1**). 6-FPOL can be synthesized in three steps starting from the commercially available hydrochloric salt of pyridoxine (vitamin B_6, **2**). First, using the procedure of Katritzky *et al.*[3], **2** was converted to 6-phenyldiazopyridoxine (**3**). Subsequent hydrogenation of the phenyldiazo moiety over palladium on carbon [4] gave the corresponding 6-aminopyridoxine (**4**) in yields up to 68%. A high degree of purity of the intermediate 6-aminopyridoxine proved to be essential for a successful fluorination of the pyridine ring to form 6-FPOL. We therefore devised a simpler, yet efficient purification method for **4** by triturating the crude 6-aminopyridine with diethyl ether (see: Experimental). The synthesis of 6-fluoropyridoxine (**1**) was finalized by a Schiemann reaction. We found that 40% HBF_4, originally described[2] as the fluoride source for this reaction, gave no isolable product. The use of 70% HF in pyridine as both reaction solvent and fluoride source gave, after neutralization of the reaction mixture and extractive work-up with diethyl ether, the desired 6-FPOL (**1**) in a pure state in yields up to 69 %.

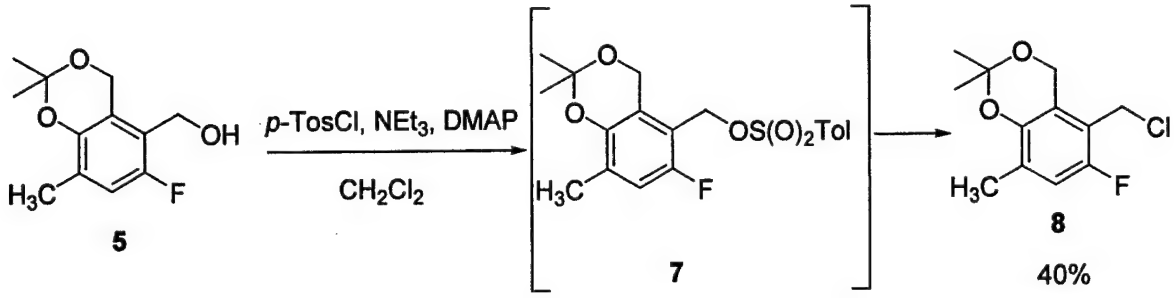


The pyridoxine skeleton offers several reactive sites for the introduction of other functional groups. We chose to use the 5-hydroxymethylene group for the introduction of an internal ^{19}F NMR reference. The synthetic potential of this site has recently been described by us[3] and others[2] taking advantage of the fact that [1] this hydroxyl group can be readily differentiated from the other hydroxyl groups in **1**. Its synthetic isolation can be achieved [4] by selectively protecting the phenolic OH and its neighboring 4-hydroxymethylene group as an acetone acetal. In addition, the position *meta* with respect to the reporter group (3-hydroxy group) and the one-carbon tether is expected to desensitize the chemical shift of the internal reference to pH changes. We decided to introduce at this position the more lipophilic (trifluoromethyl) thioether group and a 2,2,2-trifluoroacetamido moiety, respectively because of their ready synthetic accessibility.

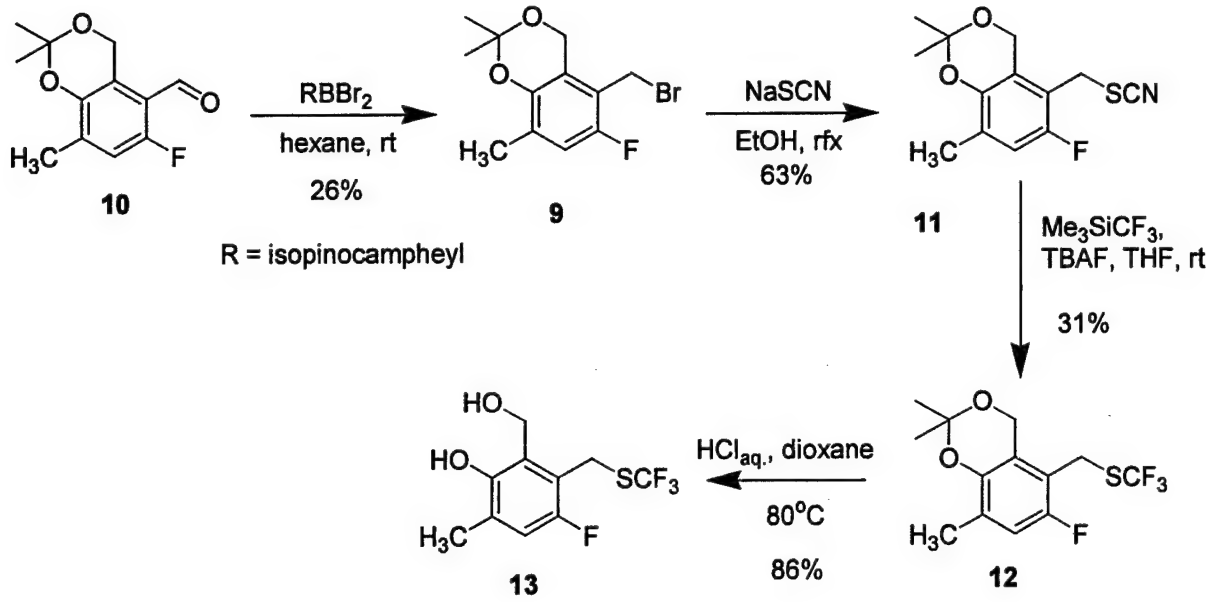
The acetonation of **1** was carried out in acetone in the presence of a five-fold excess of *p*-toluenesulfonic acid. In our hands, exclusive formation of the desired α^4,3-*O*-isopropylidene isomer **5** did not occur though as under these reaction conditions, up to 10% of the regioisomeric acetal **6** could be observed in the ^1H NMR spectrum of the crude product. The presence of regioisomer **6**, however, did not interfere with the purification of subsequent products and the crude **5** was used as such.



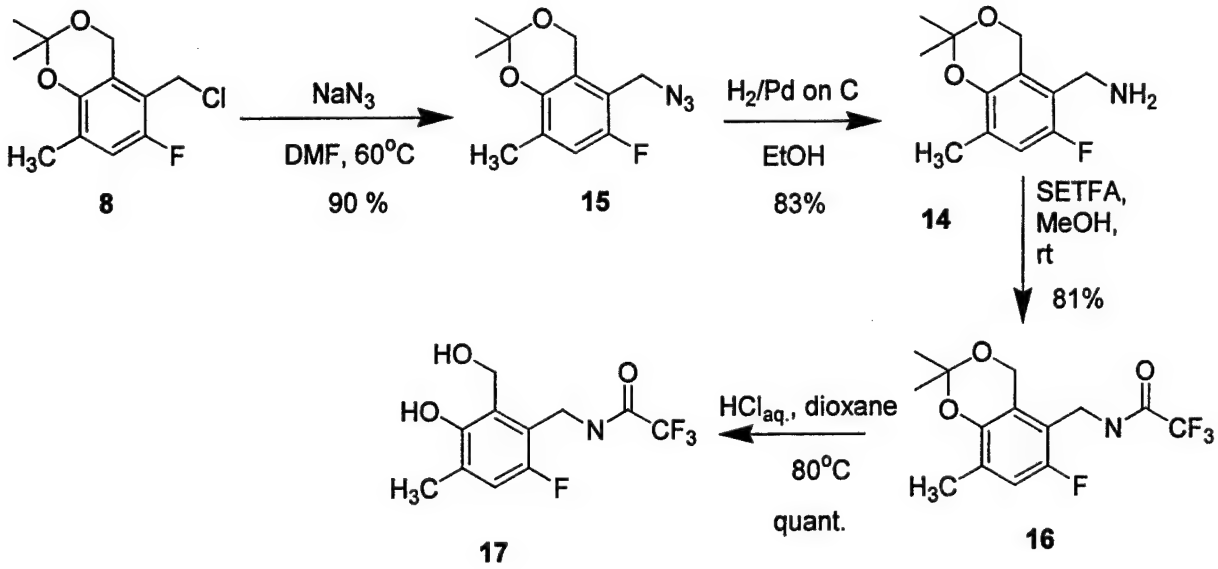
Tosylation of alcohol **5** would allow the introduction of new functionality through nucleophilic substitution reactions. Standard tosylation procedures, however, did not provide the desired sulfonic ester **7**. Instead, the use of *p*-toluenesulfonyl chloride and triethylamine as the proton acceptor in CH_2Cl_2, in the presence of a catalytic amount of DMAP, led to the isolation of pyridoxyl chloride **8** in a yield of 40%. It is believed that the highly reactive tosylate **7** serves as an intermediate that is rapidly converted to chloride **8** by HCl (or its triethylamine salt).



Substitution on chloride **8** with sodium thiocyanate in refluxing ethanol did not proceed cleanly and the resulting extensive chromatographic purification led to significant loss of product. It was decided therefor to synthesize the more reactive pyridoxyl bromide **9**, but unfortunately attempts to directly convert alcohol **5** to bromide **9** failed. The solution to this problem was found in the reductive bromination of aldehyde **10** according to a recently described procedure by Kabalka *et al.* [5]. The required aldehyde **10** was obtained by oxidation of **5** with MnO_2 according to a literature procedure[2]. Treatment of **10** with isopinocampheyl borondibromide, generated *in situ* from dibromoborane and α-pinene, furnished pyridoxyl bromide **9** in a yield of 26% after chromatography. As was anticipated, pyridoxyl thiocyanate **11** was readily obtained pure in 63% yield by reaction of **9** with NaSCN in refluxing ethanol followed by chromatographic work-up. The desired trifluoromethyl reference group was finally introduced using Ruppert's reagent[6] [6] (Me_3SiCF_3) for the conversion of thiocyanate **11** to (trifluoromethyl)thio ether **12** which was obtained pure, after chromatography in 31% yield. Final deprotection of the acetone acetal in **12** was achieved with aqueous HCl in dioxane to give our first targeted ^{19}F NMR indicator with an internal reference: **13**.



The introduction of a 2,2,2-trifluoroacetamido moiety can be achieved by acylation of pyridoxylamine **14** with (*S*)-ethyl trifluoroacetate (SETFA). Starting from pyridoxyl chloride **8**, the required pyridoxyl amine **14** could be obtained in two steps. A nucleophilic substitution on chloride **8** with NaN_3 in DMF gave pyridoxyl azide **15** in 90% yield after chromatographic purification. Subsequent hydrogenolysis of azide **15** gave **14** in 83% yield and acylation of **14** with SETFA in MeOH led to the isolation of *N*-pyridoxyl trifluoroacetamide **16** in a yield of 81%. Final deprotection of **16** with aqueous acid proceeded quantitatively to furnish **17**: a new 6-FPOL derivative with a built-in internal reference.



Titrations

The more lipophilic nature of (6-fluoropyridox-5-yl) trifluoromethyl sulfide results in poor water solubility. At neutral pH, no ^{19}F NMR signal could be observed and thus no pH/NMR titration was feasible.

The ^{19}F NMR spectrum of *N*-[6-fluoropyridox-5-yl]-2,2,2-trifluoroacetamide ( ) in water at 22 °C shows four well-separated sharp singlets corresponding to two CF_3 groups and two F substituents at the pyridoxyl ring of the two rotational isomers. Heating the sample to 90 °C does not result in a coalescence of the signals. Thus, rotation around the C-N bond of the trifluoroacetamide moiety at ambient temperatures is slow on the NMR time scale, which can be explained by the strongly electron-withdrawing nature of the CF_3 group. Because of overlap of the signal from CF_3COONa, which we routinely use as a reference for ^{19}F NMR, with the CF_3-signals KF was used as the reference. Both signals for the fluorine substituent at the pyridoxyl ring undergo an upfield shift upon an increase of the pH. The minor isomer shows a chemical shift range of 11.1 ppm between acid and base and the major isomer shows a slightly larger chemical shift range of 11.5 ppm. These observed values are an improvement with respect to the chemical shift sensitivity of 9.72 ppm for FPOL. As might be expected, the shapes of both titration curves are nearly identical, however the curves cross each other at a pH of approximately 7. Interestingly, the pK_a's of both rotational isomers are not identical. The pK_a of the minor isomer was determined at 7.7, whereas the major isomer was found to have a pK_a of 8.1. This indicates that, despite the CH_2 tether between the pyridoxyl ring and the trifluoroacetamide group, isomerization of the latter exerts a subtle, but measurable influence of the acidity of the molecule. Both signals show line broadening when the pH is close to their respective pK_a values. The chemical shifts of both CF_3 signals remain constant during the pH/NMR titration at 43.71 and 43.52 ppm for the minor and major isomer, respectively. No attempts were made to assign the ^{19}F signals to the corresponding rotational isomers.

General Remarks.

All chemicals were used as received except acetone, which was dried on K_2CO_3 prior to use. Thin-layer chromatography was carried out on Sigma/Aldrich TLC plates. Chromatographic purifications were done by flash chromatography using Merck Silica Gel, grade 9385, 230-400 mesh, 60 Å. ^1H and ^{19}F NMR were recorded on a . Chemical shifts are given in parts per million (δ), using TMS as an internal standard for ^1H NMR and CF_3COONa or KF for ^{19}F NMR. Signals are expressed as an s (singlet), d (doublet), t (triplet), m (multiplet), or br (broad).

6-Aminopyridoxine (4). To a suspension of 10.5 g (38 mmol) of phenyldiazoniumpyridoxol (**3**) in 200 ml ethanol was added 4 g of 5% Pd on carbon. The suspension was hydrogenolyzed for 16 h at room temperatures at an initial hydrogen pressure of 30 psi. The black suspension was filtered over Celite and the solvent was evaporated *in vacuo* to give an orange solid. The solid material was stirred overnight with 25 ml diethyl ether, filtered over a glass filter and dried *in vacuo* over sodium hydroxide to yield 4.8 g (68 %) of an off white solid.

6-Fluoropyridoxine (6-FPOL, 1). To 50 g HF/pyridine (70% wt HF) cooled with an ice bath, was added 15 g (81 mmol) aminopyridine (**4**) over a 30 min period. Subsequently, 7 g (101 mmol) of $NaNO_2$ was added in small portions over a 1h period. After about half of the $NaNO_2$ was added, gas evolution became less vigorous. The dark brown reaction mixture was stirred for an additional 30 min,

followed by 1.5 hours at 65°C. Initially, gas evolution resumed, subsided again after about 15 min after which all solid material had dissolved. The reaction mixture was cooled with a ice bath, diluted with 100 ml water, and carefully neutralized to a pH of 5 with sodium hydroxide pellets (**caution:** adding the hydroxide pellets too quickly and in larger amounts results in an exothermic reaction and a boiling HF-containing mixture. Alternatively, adding a concentrated sodium hydroxide solution allows a more controlled neutralization). The neutralized reaction mixture was filtered over a Büchner filter. The brown filtrate was extracted five times with 200 ml diethyl ether (with which, before each extraction, the filtration residue was thoroughly washed). The combined organic layers were dried on $MgSO_4$, filtered, and the solvent was evaporated *in vacuo* to yield 9.2 g (69 %) of a pink solid which, according to ^1H and ^{19}F NMR reported earlier, was identified as pure 6-FPOL (**1**).

6-Fluoro-α⁴,3-*O*-isopropylidenepyridox-5-yl chloride ( ). To a solution of 3.74 g (16.4 mmol) 6-fluoro-α⁴,3-*O*-isopropylidenepyridoxol in 12 ml dry CH_2Cl_2 were added 5.7 g (30 mmol) *p*-toluenesulfonyl chloride, 10 ml triethylamine, and 100 mg DMAP. An exothermic reaction ensued and the reaction mixture was cooled for 10 min with an ice bath. A precipitate eventually appeared and the reaction mixture was stirred for 16 h at room temperature. The dark brown reaction mixture, diluted with 75 ml water and an additional 150 ml CH_2Cl_2, was transferred to a separating funnel. The separated organic layer was dried on $MgSO_4$, filtered, and evaporation of the solvent *in vacuo* gave a brown mass which was purified with flash chromatography (eluent: 10% EtOAc in hexane) to yield 1.6 g (40 %) of a white solid. ^1H NMR ($CDCl_3$) δ 1.56 (s, 6H), 2.36 (s, 3H), 4.50 (s, 2H), 4.97 (s, 2H). MS (m/z)

6-Fluoro-α⁴,3-*O*-isopropylidenepyridox-5-yl bromide ( ). To a solution of 1.54 g (6.8 mmol) of 6-fluoro-α⁴,3-*O*-isopropylidenepyridox-5-al in 25 ml hexane under argon, was added, through a syringe, a freshly prepared solution of isopinocampheyl borondibromide (by refluxing under argon, for 5 h, 7.5 ml of a 1.0 M solution of $HBBr_2 \bullet Me_2S$ in CH_2Cl_2 with 1.2 ml α-pinene) at room temperature. A white precipitate immediately formed and stirring was continued overnight. The reaction mixture was filtered over a paper filter and the reaction flask was rinsed twice with 75 ml CH_2Cl_2. The combined organic layers were washed once with 100 ml water, dried on $MgSO_4$, filtered, and the solvent was evaporated *in vacuo*. The crude product was further purified with flash chromatography (eluent: 10% EtOAc in hexane) to give 514 mg (26 %) of a white solid. ^1H NMR ($CDCl_3$) δ 1.55 (s, 6H), 2.36 (s, 3H), 4.34 (s, 2H), 4.92 (s, 2H).

6-Fluoro-α⁴, 3-*O*-isopropylidenepyridox-5-yl thiocyanate ( ). To a solution of 514 mg (1.9 mmol) of 6-fluoro-α⁴, 3-*O*-isopropylidenepyridox-5-yl bromide ( ) in 5 ml ethanol, was added 180 mg (2.2 mmol) NaSCN. The mixture was refluxed overnight. TLC (eluent: 10% EtOAc in hexane) confirmed complete conversion of the starting material. The solvent was evaporated *in vacuo* and the crude product was purified by flash chromatography (eluent: 10% EtOAc in hexane) to give 316 mg (63 %) of a white solid. ^1H NMR ($CDCl_3$) δ 1.57 (s, 6H), 2.38 (s, 3H), 4.06 (s, 2H), 4.97 (s, 2H). MS (m/z)

(6-Fluoro-α⁴, 3-*O*-isopropylidenepyridox-5-yl) trifluoromethyl sulfide ( ). To 4.5 ml of a 0.5 M solution of Me_3SiCF_3 in THF (2.25 mmol) was added 300 mg (1.1 mmol) 6-fluoro-α⁴, 3-*O*-isopropylidenepyridox-3-yl thiocyanate ( ) and 0.2 ml of a 1 M solution of TBAF in THF (Aldrich). The reaction mixture was stirred overnight. The solvent was evaporated *in vacuo* and the crude product was

purified by flash chromatography (eluent: 10% EtOAc in hexane) to give 108 mg (31 %) of a colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.55 (s, 6H), 2.35 (s, 3H), 3.90 (s, 2H), 4.91 (s, 2H). MS (m/z)

(6-Fluoropyridox-5-yl) trifluoromethyl sulfide ( ). To a solution of 108 mg (0.34 mmol) of (6-fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl) (trifluoromethyl) sulfide ( ) in 3 ml dioxane was added 1.5 ml 1 M HCl. The reaction mixture was stirred at 80 °C for 3 h and subsequently neutralized with aqueous K_2CO_3. The aqueous mixture was extracted 3 times with 10 ml EtOAc. The combined organic layers were dried on $MgSO_4$, filtered, and the solvent was evaporated *in vacuo* to give 80 mg (86 %) of a white solid.
^{1}H NMR (dmso-d_6) δ 2.31 (s, 3H), 4.33 (s, 2H), 4.72 (d, J = 4 Hz, 2H), 5.97 (d, 1H), 9.15 (br, 1H). MS (m/z)

6-Fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl azide ( ). To a solution of 780 mg (3 mmol) 6-fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl chloride (**8**) in 5 ml DMF was added 650 mg (10 mmol) NaN_3. The reaction mixture was stirred under an argon atmosphere for 16 h, allowed to cool to room temperature, and poured into 50 ml water. The aqueous layer was extracted three times with 50 ml EtOAC, and the combined organic layers were washed three times with 50 ml water. The organic layer was dried on $MgSO_4$, filtered, and the solvent was evaporated *in vacuo*. Flash chromatography (eluent: 10% EtOAc in hexane) gave 713 mg (90%) of a colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.55 (s, 6H), 2.36 (s, 3H), 4.27 (s, 2H), 4.88 (s, 2H).

6-Fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl amine ( ). To a solution of 700 mg (2.7 mmol) of 6-fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl azide ( ) in 50 ml EtOH was added 400 mg 5% Pd on C. The reaction mixture was hydrogenolyzed at 20 psi for 16 h at room temperature. The reaction mixture was filtered over Celite and evaporation of the solvent in vacuo gave pure amine in an 83% yield in the form of a slowly solidifying oil. ^{1}H NMR ($CDCl_3$) δ 1.52 (s, 6H), 2.33 (s, 3H), 3.73 (s, 2H), 4.97 (s, 2H).

***N*-[6-Fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl]-2,2,2-trifluoroacetamide ( ).** To a solution of 250 mg (1.1 mmol) pyridoxyl amine ( ) in 5 ml methanol was added a solution of 180 µl SETFA (1.3 mmol) in 3 ml methanol. The reaction mixture was stirred overnight. TLC (eluent: 25 % EtOAc in hexane) indicated complete conversion of the starting material. The solvent was evaporated *in vacuo* and the crude product was purified by flash chromatography (eluent: 25 % EtOAc in hexane) to give 292 mg (81%) of a white solid. ^{1}H NMR ($CDCl_3$) δ 1.54 (s, 6H), 2.35 (s, 3H), 4.36 (d, J = 6.0 Hz, 2H), 5.03 (s, 2H), 6.8 (br, 1H).

N-[6-Fluoropyridox-5-yl]-2,2,2-trifluoroacetamide ( ). To a solution of 104 mg (0.32 mmol) of *N*-[6-fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl]-2,2,2-trifluoroacetamide ( ) in 5 ml 1,4-dioxane, was added 1 ml 1M HCl. The clear solution was stirred for 4 h at 80 °C. After the reaction mixture was allowed to cool to room temperature, the pH was adjusted to 7 with 1 M NaOH. The solvents were evaporated *in vacuo* and the remaining white solid was triturated with 10 ml EtOAc and filtered. The filtrate was dried, filtered, and the solvent was evaporated *in vacuo* to leave 86 mg (100 %) of a white solid. ^{1}H NMR (dmso-d_6) δ 2.28 (s, 3H, major isomer), 4.41 (br, 2H), 4.68 (s, 2H, major isomer).

Acknowledgments

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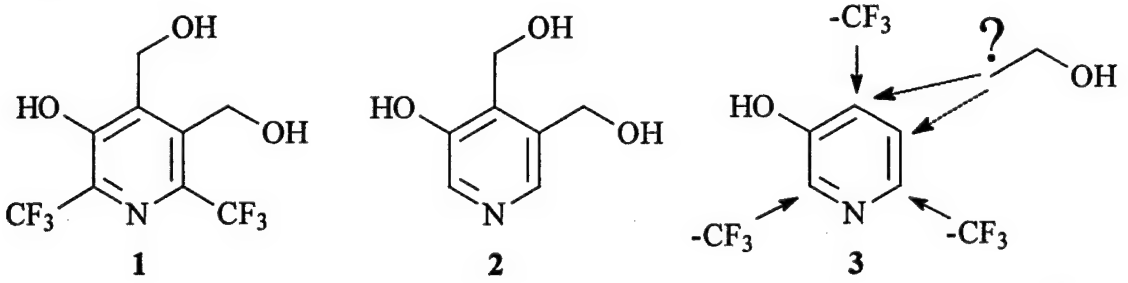
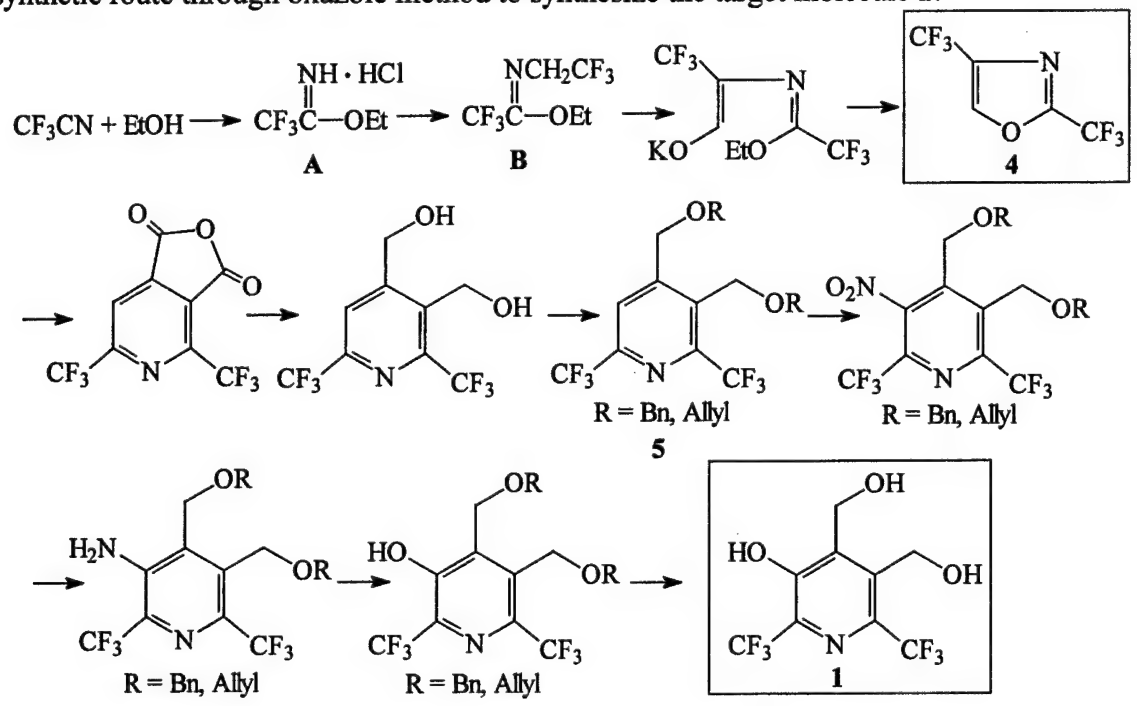
Appendix 4

Attempted Syntheses of diCF_3 pyridoxol pH Indicators

I. For the target molecule **1**, we initially hoped to synthesize it from 3,4,5-trisubstituted pyridine **2**, but **2** is not commercially available. The commercial pyridine derivative **3** can be used as starting material by introducing $-CF_3$ groups through reported methods although there are three possibly introduced sites. However, no methods or procedures so far can successfully construct the $-CH_2OH$ groups at 4,5 positions.

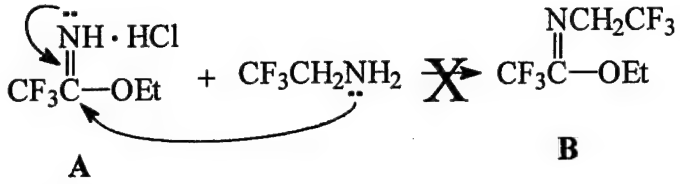


After looking up the references from three aspects: pyridine ring construction, pyridine derivatives synthesis and synthetic methods and on the basis of retro-synthesis analysis, we proposed a novel synthetic route through oxazole method to synthesize the target molecule **1**:



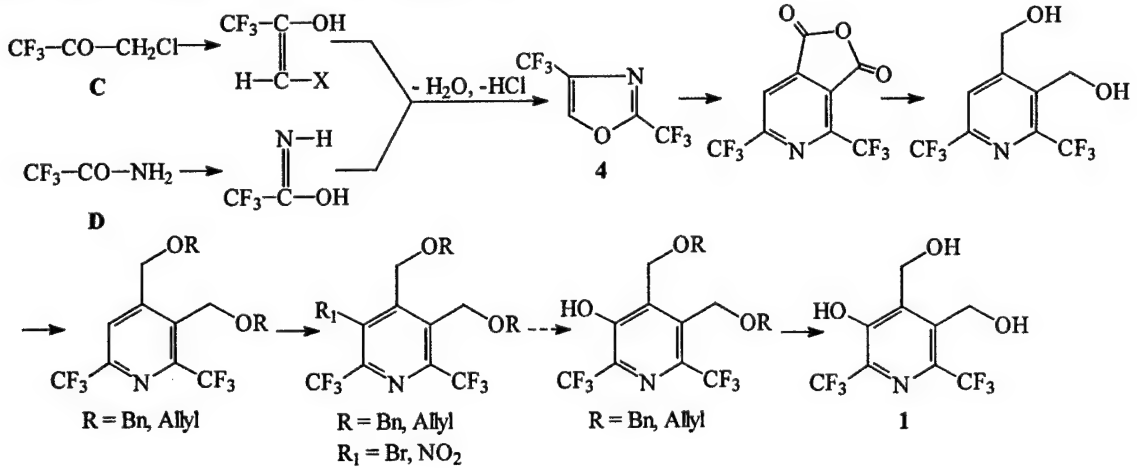
After many attempts, we successfully got the key intermediate **A** and found a facile procedure by using the very highly toxic starting material CF_3CN, but failed to obtain important synthon **B**. Probably because the presence of strong electron-withdrawing group $-CF_3$ made the electron-pairs on nitrogen atoms move to the CF_3 and result that the electron-pairs leave with much high difficulty (in

compound **A**) and have very low nucleophilicity (in $CF_3CH_2NH_2$) to attack on the carbon and cause replacement reaction.

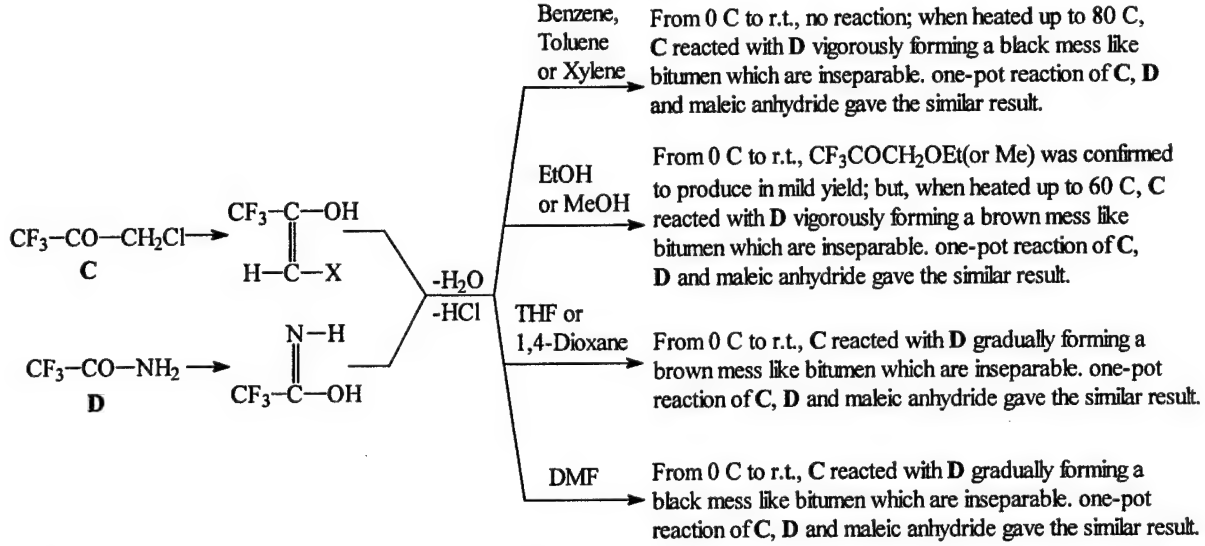


A **B**

Thus, we designed another similar approach still by oxazole method but from different starting materials **C** and **D** avoiding the above reaction.

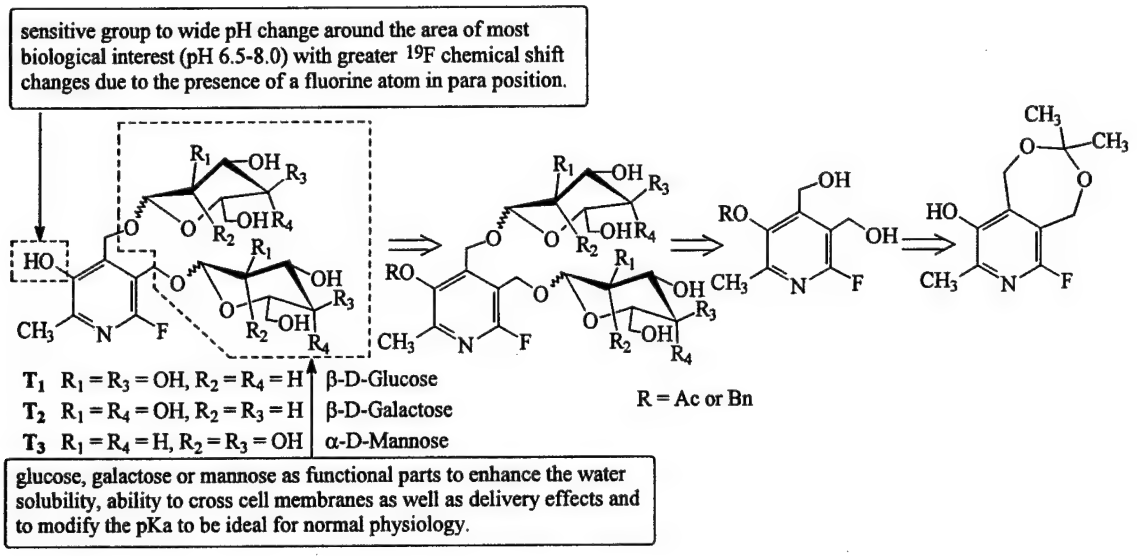


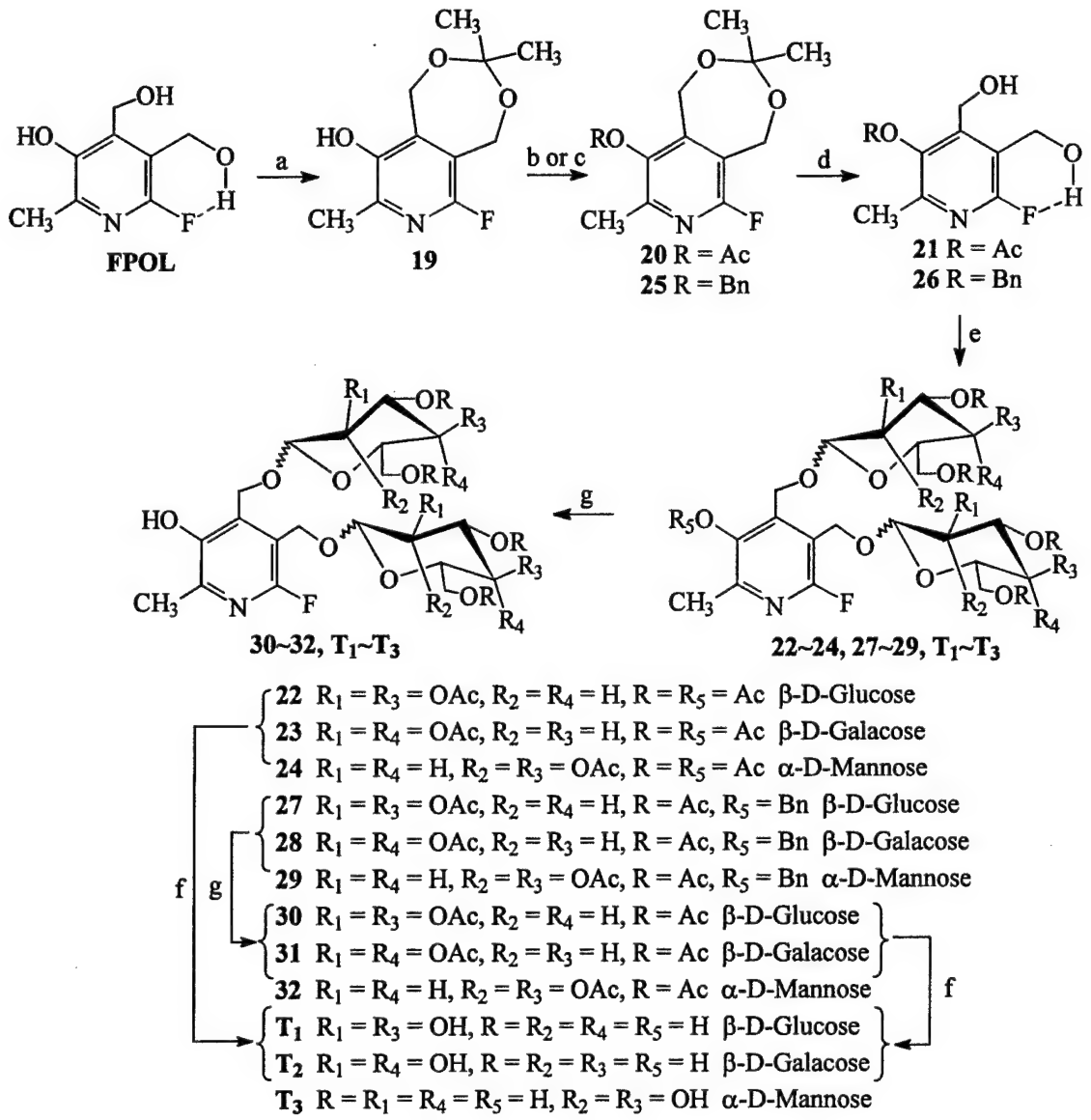
From this novel procedure, the oxazole intermediate **4** would be synthesized by one-step reaction. However, when we tried to synthesize it, we met some problems indicating that the reaction of **1** and **2** is very complex due to the presence of CF_3- groups:



The introduction of a trifluoromethyl group into an organic compound can bring about remarkable changes in the physical, chemical and biological properties. Actually, organic fluorinated

compounds synthesis or chemistry up to now are real challenges, which not like normal organic chemistry can predict the reaction direction or products. If any major product could be separated in the above new reaction between **C** and **D**, it would be of great value in organic fluorinated chemistry. Systematic review of the literature and our own experience suggest that the di CF_3POL is currently not a feasible target

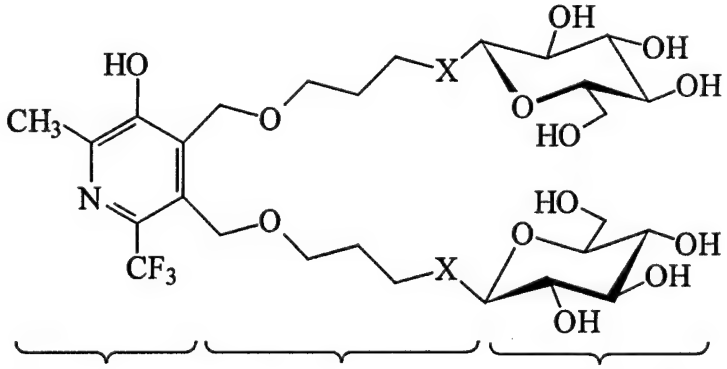




The measurements of pH *vs.* ^{19}F NMR chemical shift of T_1~T_3 in diverse solvents and temperatures show that, after glycosylation at 4,5-positions, T_1~T_3 behave like **6-FPOL**, the pH dependent ^{19}F NMR chemical shifts are still essentially independent of the presence of metal ions across the whole pH range and of the temperature in the range 25~37°C. But, T_1~T_3 exhibit more ideal *pKa* for normal physiology by the modification of glycosylation at 4,5-positions. Applications of T_1~T_3 for measurement of both intra- and extracellular pH are currently under investigation. In addition, by comparing the structures of T_1~T_3, we found that glucose, galactose or mannose in *α*- or *β*-configurations linked at 4,5-positions have only very minor effect on the acidities and ^{19}F NMR/pH properties.

As expected, 6-trifluoromethyl pyridoxol **CF_3POL** provides higher gain in signal to noise over our previous prototype agents (**6-FPOL**) and its derivatives. The *pKa* of 6.82 makes it much better for investigations of breast cancer, though the chemical shift between acid and base conditions, of 1.64 ppm is much less than that of 6-fluoropyridoxol and its derivatives.

Our experiences on the syntheses and evaluation of 6-fluoropyridoxol and its derivatives have shown that modification of either 4- or 5-hydroxymethyl moiety produces modification of the pK_a. In addition, people have found that cell-permeant, or translocating compounds are characterized by their ability to breach the cell membrane and deliver attached cargoes without causing lethal membrane disruption, and are dependent on their hydrophobicity and hydrophilicity balance. Based on these findings, we thought of applying these strategies to design two molecules having the following characteristics with the end on the ability to cross cell membranes: (a) the aglycone used was 6-trifluoromethyl pyridoxol as core structure; (b) the non-ionic, biocompatible component carbohydrates as surfactants to enhance the water solubility, ability to cross cell membranes as well as delivery effects; (c) the core and the carbohydrates were linked through two different length and nature aliphatic alkoxy spacers, which have been already studied and have given promising results on playing a decisive role for the accessibility to molecular recognition events and affecting ability into cell membranes as well as adjusting the amphiphilic nature of the molecules.

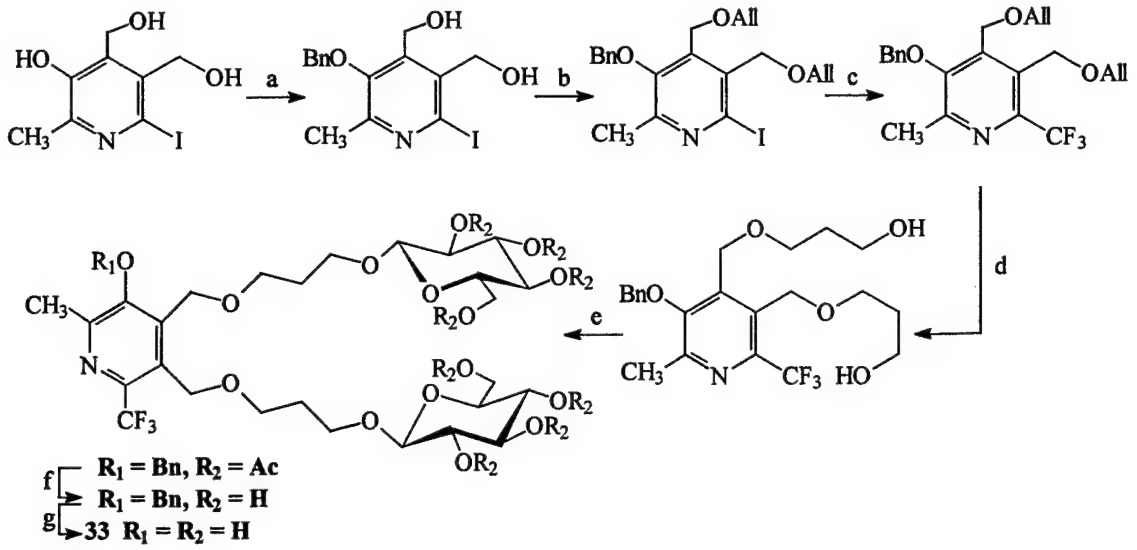


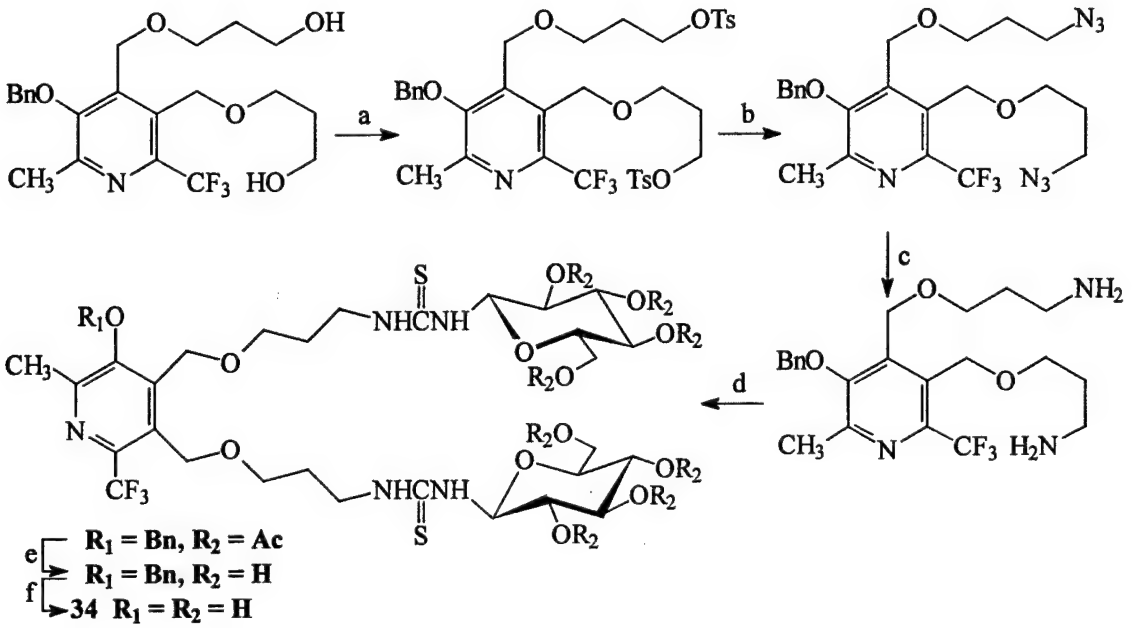
pH sensitive moiety alkoxy spacers **specific markers**

33 X = O; **34** X = NHC(S)NH

For the synthesis of the designed target molecules **33** and **34**, we started with **6-IPOL** as the choice, and the main problems were the protecting groups and the selectivity of fixation between the

different hydroxyl groups present in the structure of **6-IPOL**. Herein, we successfully developed an efficient approach for regioselective protection of 3-phenolic hydroxyl group.





While the current grant has finished, we propose to continue this work under the auspicies of the P20.

Appendix 5

Design, Synthesis and Characteristics of Novel Carbohydrate Clustering pH indicators based on Fluorinated Vitamin B_6 for *in vivo* and Non-invasive Detection

Jianxin Yu Ralph P. Mason*

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Introduction

pH is considered to play a significant role in cellular regulation[1]. In particular, the pH gradient between the interstitial and intracellular compartments is involved in many regulatory processes, and strongly influences drug uptake. Thus, the measurement of pH in tumors promises to provide insight into developmental processes and prognostic information regarding therapeutic outcome. Many approaches for pH measurement have been developed based on weak acid/base distributions, microelectrodes, fluorescent probes radiolabelling and NMR spectroscopy[1,2]. The NMR methods provide the potential advantages of continuous, repetitive, nondestructive measurements of pH *in vivo* and simultaneous detection of pH in several compartments. We have previously demonstrated that the application of 6-fluoropyridoxol (**1**) to simultaneously measure both intra- and extracellular pH providing well resolved resonances exhibiting not only exceptional sensitivity to changes in pH with ~10ppm acid/base ^{19}F NMR shift in whole blood and the perfused rat heart[1,3-5], but negligible response to changes in metal ion concentrations, the presence of proteins, or variation in temperature in titration process. With the aims at increasing the water solubilities, abilities to cross cell membranes as well as delivery effects and modifying the *pKa* of **1** to be ideal for normal physiology, in this work, we report the design, synthesis and ^{19}F NMR characteristics of three novel carbohydrate clustering pH indicators $\mathbf{T_1}$~$\mathbf{T_3}$ using fluorinated vitamin B_6 as scaffold (Scheme 1).

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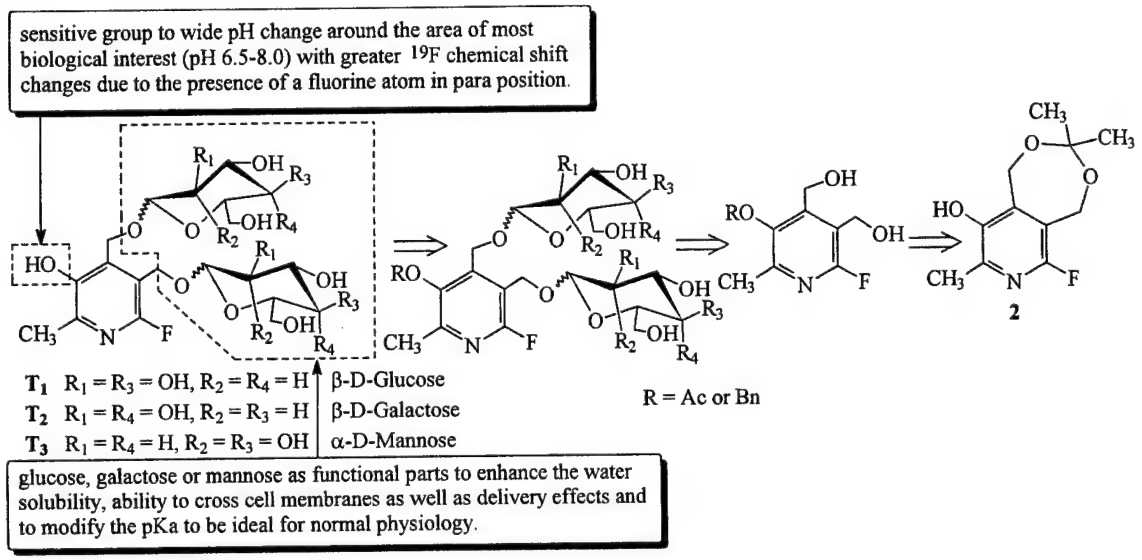
Results and Discussion

6-Fluoropyridoxol **1** has three available positions for modification. As shown by us and others[6,7], introduction of a fluorine atom *para* to the 3 phenolic group provides a titratable ^{19}F NMR signal, which is highly sensitive to changes in pH. We have further shown that modification of the 4- and 5-position hydroxymethyl moieties produces modification of the pK_a with relatively minor changes in chemical shift and chemical shift range. So, to our purpose, we believed that the free 3 phenolic group could be sensitive to wide pH changes around the area of most biological interest (pH 6.5-8.0) with greater ^{19}F chemical shift changes due to the presence of a *para* fluorine atom, while the 4, 5 hydroxymethyl groups are modified by glycosylations to anticipate carbohydrates as functional parts to modify the pK_a to be ideal for normal physiology and to enhance the water solubility, ability to cross cell membranes as well as delivery effects, because the substitution by sugars could provide the molecule with polar hydroxyl groups and possibly lead the conjugate to the tumor cell surface target and increase the ability to penetrate cell walls through the specific binding to its receptor[8]. This specificity also suggests a potential utility of the designed saccharide pH indicator molecules could be delivered directly to tumor cells. According to the retro-synthesis analysis, we proposed an approach through protected intermediates **2** as key synthon for synthesis of target compounds **T_1~T_3** (Scheme 1).



Scheme 1. The proposed synthetic routes for **T_1~T_3**.

6-Fluoro-α^4, α^5-isopropylidenepyrodoxol **2** was ~~ever~~ found as minor by-product in our previous preparation of 6-fluoro-3, α^4-isopropylidenepyrodoxol. We assumed that the reasons for producing **2** in very low yield were mainly due to the formation of stable hydrogen bond between α^5-OH and 6-F restricting the activity of α^5-OH in the acetonation, and in the reverse, the presence of the fluorine atom in the 6-position made its *para* 3 phenol more active as prior hydroxyl group participating in the aldol addition. So, if acetonation reaction was hoped to occur regioselectively on 4, 5 hydroxymethyl groups, the hydrogen bond between α^5-OH and 6-F must be broken and the higher activity of 3 phenol be limited in the aldol condensation. Through our attempts by employing different kind and concentration acids as catalysts, 2% H_2SO_4 acetone solution proved to be viable for the synthesis of **2** in a yield of 26%. The regioselectivity of the acetonation reaction was confirmed by analyzing ^{1}H-NMR spectra of **2** and 6-fluoro-3, α^4-isopropylidenepyrodoxol, in which 5-CH_2 signal of **2** appeared at 5.03ppm as singlet and 6-fluoro-3, α^4-isopropylidenepyrodoxol at 4.97ppm but as doublet (J=1.2Hz) due to the coupling of 5-OH.

To employ ~~more~~ less step of reactions, we initially designed the acetylation as protecting strategy so that the 3-O-acetyl group could be removed conveniently together with 2, 3, 4, 6-tetra-*O*-acetyl groups of glucose, mannose and galactose during the conversion of **8~10** to $\mathbf{T_1}$~$\mathbf{T_3}$. Acetylation of **2** as usual work up gave rise to 3-O-acetyl-6-fluoro-α^4, α^5-isopropylidenepyrodoxol **3** in high yield. Cleavage of acetonide **3** for synthesis of the key intermediate **4** was achieved, but the results were much complicated and **4** was obtained in only quite low yield (≤30%), on whatever any hydrolysis conditions used such as 80% AcOH, 1% HCl or 90% CF_3CO_2H in MeOH, CH_2Cl_2 or 1,4-dioxane in different temperature different. Careful purification and characterization of the products as α^4-*O*-acetyl-6-fluoropyrodoxol (20%), α^5-*O*-acetyl-6-fluoropyrodoxol (15%) and 6-fluoropyrodoxol (35%) confirmed that the migration and deactylation reactions occurred accompanying the acidic hydrolysis of α^4, α^5-isopropylidene group of **3**, presumably due to the presence of 6-fluorine atom resulting in Ac-O_3 bond more active and sensitive. Eventually, although we successfully obtained $\mathbf{T_1}$~$\mathbf{T_3}$ as designed in Figure

1 through glycosylation and followed by deacetylation of **8~10**, the total yields of $\mathbf{T_1}$~$\mathbf{T_3}$ are much too low (5~7%).

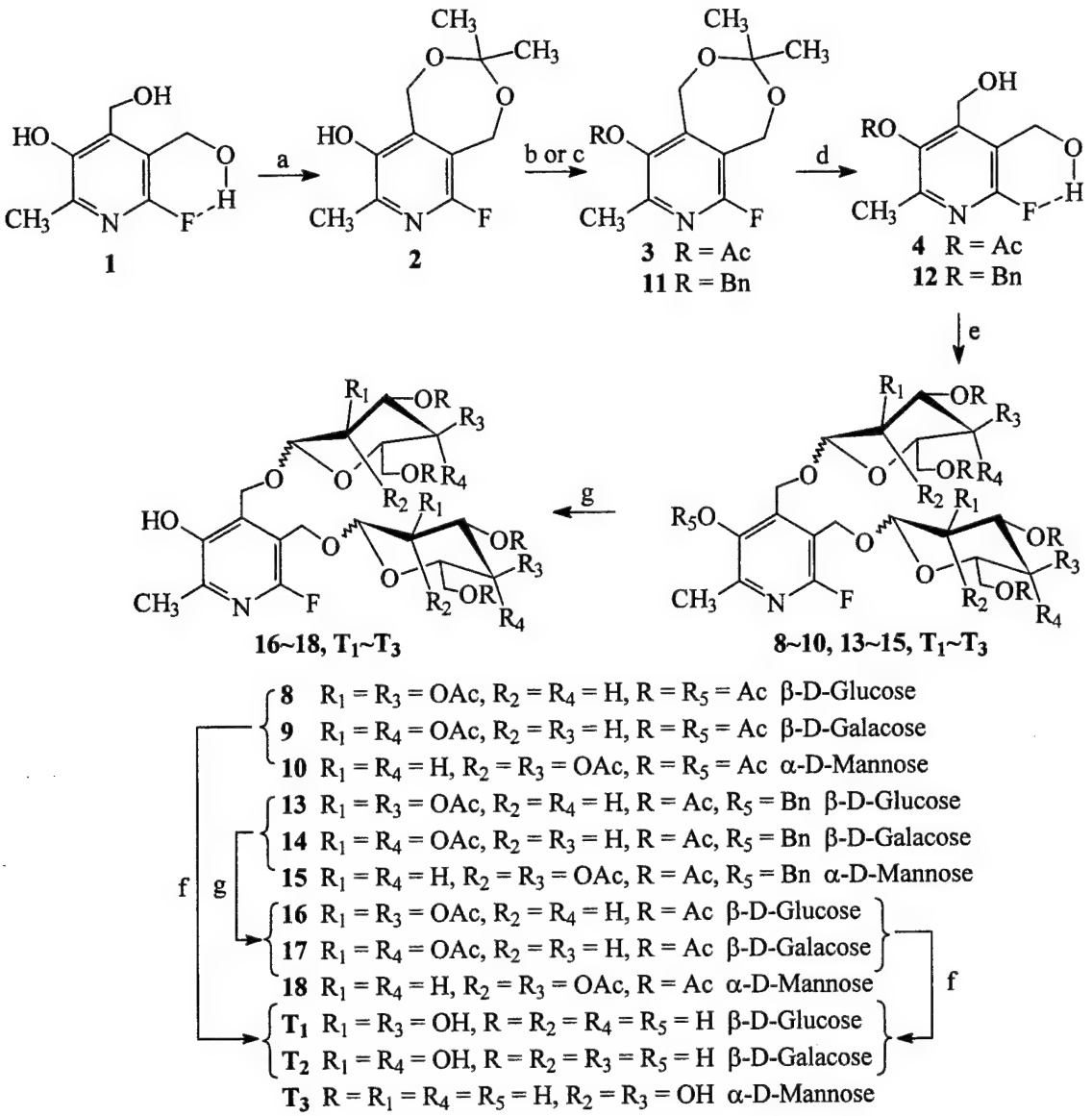


Figure 1. Reagents and conditions: (a) 2% H_2SO_4, acetone, r.t. 4~5hr, 26%; (b) Ac_2O-Pyridine, 0°C→r.t., 24hr, 94%(→**3**); (c) NaH, benzyl bromide(1.1 equiv.), DMF, r.t., 2hr, 94%(→**11**); (d) CH_2Cl_2-CF_3CO_2H, reflux, 24hr, 30%(→**4**) or MeOH-CF_3CO_2H, reflux, ~6hr, 94%(→**12**); (e) 2,3,4,6-tetra-*O*-acetyl-*α*-D-glucopyranosyl bromide(**5**), 2,3,4,6-tetra-*O*-acetyl-*α*-D-galacopyranosyl bromide(**6**) or 2,3,4,6-tetra-*O*-acetyl-*α*-D-mannopyranosyl bromide(**7**), $Hg(CN)_2$, 4Å M.S., CH_2Cl_2, r.t., 12hr, 90%(→**8**), 86%(→**9**), 70%(→**10**) or 95%(→**13**), 93%(→**14**) or 90%(→**15**), respectively; (f) NH_3-MeOH, 0°C→r.t., 24hr, 90~97%; (g) H_2, Pd/C, r.t., 12hr, quantitative yields.

Thus, we turned our attention to the stable protecting procedure. Benzylation of **2** gave 3-*O*-benzyl-6-fluoro-α^4, α^5-isopropylidenepyrodoxol **11** in a yield of 94%. Hydrolysis of **11** in 90% CF_3CO_2H-MeOH solution for synthesis of **12** proceeded in high yield(94%). Treatment of **12** respective with 2, 3, 4, 6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **5**, 2, 3, 4, 6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **6** and 2, 3, 4, 6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide **7** under the conditions of the Koenigs-Knorr glycosylation method afforded 3-O-benzyl-α^4, α^5-di-O-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyrodoxol **13**(95%), 3-O-benzyl-α^4, α^5-di-O-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galacopyranosyl)-6-fluoropyrodoxol **14**(93%) and 3-O-benzyl-α^4, α^5-di-O-(2, 3, 4, 6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyrodoxol **15**(90%). The $\delta_{H\text{-}1'}$ at 4.4~5.4ppm in well-resolved doublet with J=~8.0Hz for **8~9**, **13~14** and $\delta_{C\text{-}1'}$ at ~100ppm demonstrated that the glucose and galactose were in the β-configuration, the $\delta_{H\text{-}1'}$ at ~5.4ppm (J=2.4Hz) and $\delta_{C\text{-}1'}$ at 99ppm for **10**, **15** verified that mannose was in α-configuration. After glycosylation at 4,5-position, it was found that the chiral glycoses made two equally geminal protons of 4 or 5-CH_2 become magnetically non-equivalent and split into two doublets.

Hydrogenation of **13~15** in the presence of Pd/C furnished **16~18**, followed by deacetylation in NH_3/MeOH from 0°C to room temperature accomplished the target molecules $\mathbf{T_1}$~$\mathbf{T_3}$ all in near quantitative yields in these two steps of reactions. But, the overall yields for $\mathbf{T_1}$~$\mathbf{T_3}$ through the five-step were only of 19%~20%, with limiting step in the selective α^4, α^5-isopropylidene group preparation procedure.

Comparing the three hydroxyl groups in compound **1**, we know that they are all actively reactive groups, but have much difference in acidities. The acidic 3 phenolic hydroxyl group should be easily converted into the monoanion form and could be transferred across the dichloromethane-aq. alkali interface with the aids of tetrabutylammonium cation to attack benzyl bromide affording the expected 3- solo benzylated product. In order to improve the above five-step process, an alternative approach of benzyl bromide reacting with compound **1** directly has been developed. By controlling the amount and the dropping speed of benzyl bromide as well as the pH of the reaction mixture, benzyl bromide (1.1 equiv.) was added dropwise over a period of 4~5h

to the well-stirred reaction mixture of compound **1** in a dichloromethane-aqueous biphasic system (pH 10~11) employing tetrabutylammonium bromide (TBAB) as the phase-transfer catalyst, 3-O-benzyl-6-fluoropyrodoxol **12** was isolated as major product in 76% yield. Condensation of **12** with **5**, **6** or **7** accomplished **13~15** in satisfactory yields, which were subjected to hydrogenation and deacetylation procedures giving the target compounds $\mathbf{T_1}$~$\mathbf{T_3}$ in 62~71% overall yields over four steps (Figure 2).

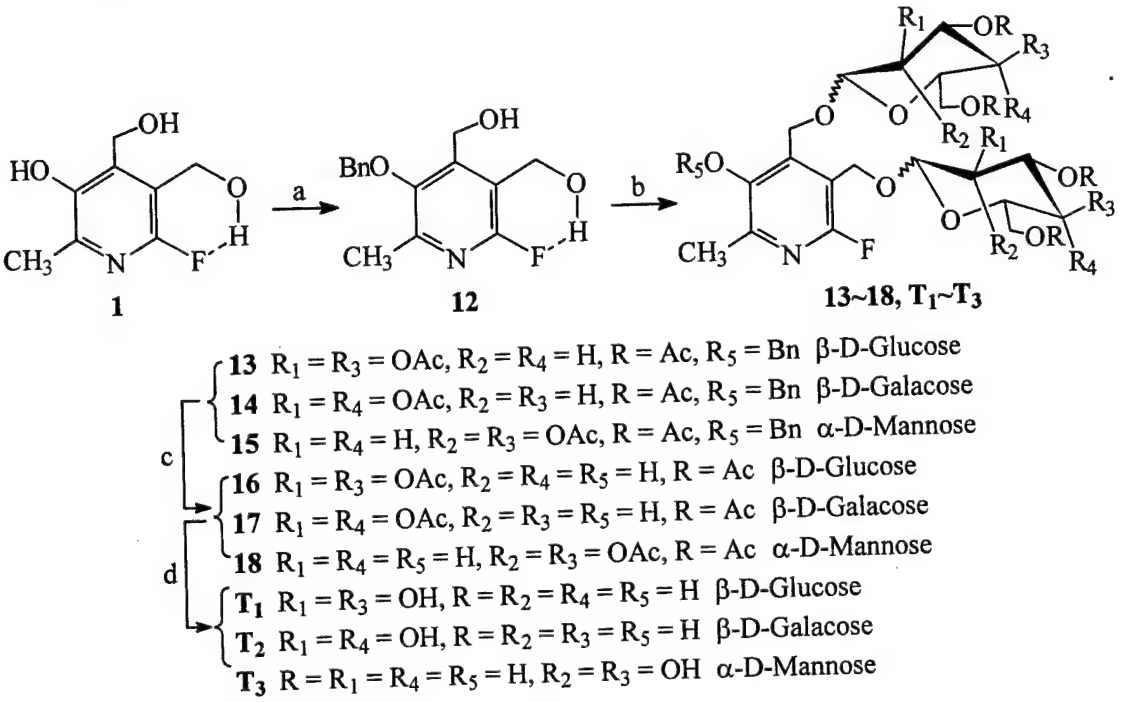


Figure 2. Reagents and conditions: (a) Benzyl bromide (1.1 equiv.), CH_2Cl_2-H_2O, pH 10~11, 50°C, TBAB, 5~6hr, 76%; (b) 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**5**), 2,3,4,6-tetra-*O*-acetyl-α-D-galacopyranosyl bromide (**6**) or 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide (**7**), $Hg(CN)_2$, 4Å M.S., CH_2Cl_2, r.t., 12hr, 95%(→**13**), 93%(→**14**) or 90%(→**15**), respectively; (c) H_2, Pd/C, r.t., 12hr, quantitative yields; (d) NH_3-MeOH, 0°C→r.t., 24hr, 97%(→ $\mathbf{T_1}$), 95%(→$\mathbf{T_2}$) or 91%(→$\mathbf{T_3}$), respectively.

In conclusion, the synthetic strategies described here provide very efficient methods to stereo- and regioselectively synthesize saccharide-cluster derivatives using 6-fluoropyrodoxol **1** as a scaffold for the ^{19}F NMR-based *in vivo* pH indicators screen.

As expected, $\mathbf{T_1}$~$\mathbf{T_3}$ all show the ~~perfect~~ excellent aqueous solubility. The ^{19}F NMR spectra of $\mathbf{T_1}$~$\mathbf{T_3}$ were obtained over 1~13 pH range. From the titration curve, the coefficients *pKa*, $\delta_{(acid)}$ and $\delta_{(base)}$ of the Henderson-Hasselbach equation were determined. The chemical shift differences between acid and base forms of $\mathbf{T_1}$~$\mathbf{T_3}$ are reported in the

Table 1 along with the chemical shift sensitivities, derived from slope analysis of the pH/chemical shift graphs, and an estimation of the useful pH ranges. The NMR spectra indicate that T_1~T_3 exhibit single sharp ^{19}F NMR resonance in aqueous solution. Their ^{19}F NMR chemical shifts are pretty sensitive to pH with the range of ~11ppm. Figure 3 is the titration curve of T_2 in saline at 37°C. Figure 4 is the ^{19}F NMR spectra of T_2 in acid and base.

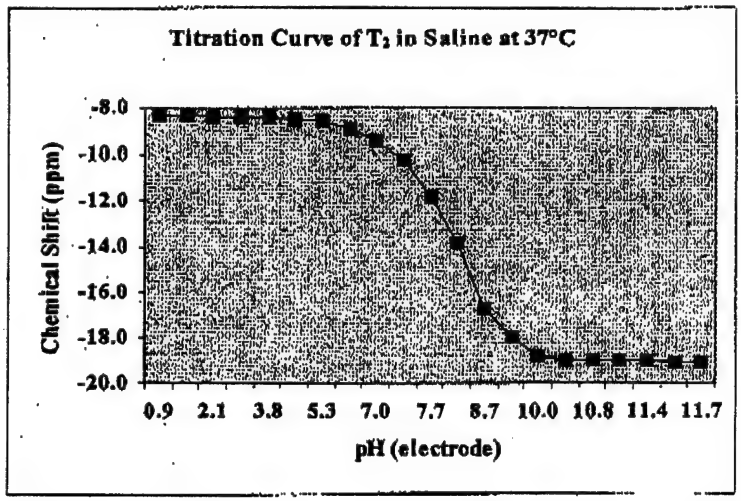


Fig. 3 The titration curve of T_2 in saline at 37°C

Table 1 Acidities and ^{19}F NMR/pH properties of T_1~T_3.

Compd.	pKa	^{19}F NMR				
		$\delta_{(acid)}$	$\delta_{(base)}$	$\Delta\delta$	ppm/pH unit	pH range
T_1	7.7	-8.06	-18.96	10.90	2.00	5.20~9.90
T_2	7.8	-8.34	-19.03	10.69	1.90	5.32~10.01
T_3	8.0	-7.86	-18.12	10.26	1.81	5.01~9.85

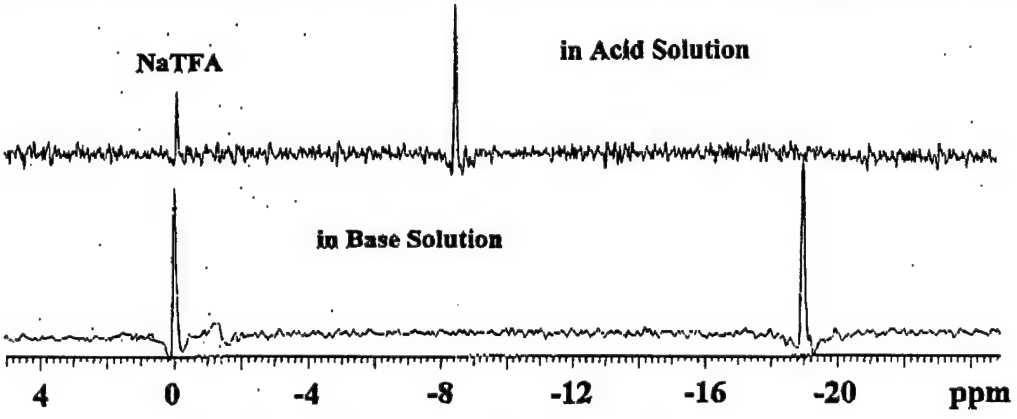


Fig. 4 The ^{19}F NMR spectra of T_2 in acid and base solution

The measurements of pH *vs.* ^{19}F NMR chemical shift of $\mathbf{T_1}$~$\mathbf{T_3}$ in diverse solvents and temperatures show that, after glycosylation at 4,5-positions, $\mathbf{T_1}$~$\mathbf{T_3}$ behave like **1**, the pH dependent ^{19}F NMR chemical shifts are still essentially independent of the presence of metal ions across the whole pH range and of the temperature in the range 25~37°C. But, $\mathbf{T_1}$~$\mathbf{T_3}$ exhibit more ideal *pKa* for normal physiology by the modification of glycosylation at 4,5-positions. The deep applications of $\mathbf{T_1}$~$\mathbf{T_3}$ for measurement of both intra- and extracellular pH are currently under investigation. In addition, by comparison the structures of $\mathbf{T_1}$~$\mathbf{T_3}$, we found that glucose, galactose or mannose in α- or β-configurations linked at 4,5-positions have only very minor effect on the acidities and ^{19}F NMR/pH properties. On the basis of these results, the synthesis of more complex saccharide-clustering glycosides is in progress.

Experimental

General methods---- All NMR spectra were recorded on a Varian MERCURY 400 spectrometer (400MHz for ^1H, 100MHz for ^{13}C, 376MHz for ^{19}F) with $CDCl_3$, acetone-d_6 or DMSO-d_6 as solvents, ^1H and ^{13}C chemical shifts are referenced to TMS as internal standard, ^{19}F to a dilute solution of sodium trifluoroacetate (NaTFA) in a capillary as external standard. Chemical shifts are given in ppm. All compounds were characterized by acquisition of ^1H, ^{13}C, DEPT, ^1H-^1H COSY or NOESY experiments at 25°C, and ^{19}F spectra were performed at 25~37°C. Of the two magnetically nonequivalent geminal protons of sugar parts at C-6, the one resonating at lower field is denoted H-6a and the one at higher field is denoted H-6b. Mass spectra were obtained by positive and negative ESI-MS using a Micromass Q-TOF hybrid quadrupole/time-of-flight instrument (Micromass UK Ltd). 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**5**), 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**6**) and 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide (**7**) were purchased from the Sigma Chemical Company. Reactions requiring anhydrous conditions were performed under nitrogen or argon. $Hg(CN)_2$ was dried before use at 50°C for 1h, CH_2Cl_2 was dried over Drierite, and acetonitrile was dried on CaH_2 and kept over molecular sieves under nitrogen. Solutions in organic solvents were dried with anhydrous sodium sulfate, and concentrated *in vacuo* below 45°C. Column chromatography was performed on silica gel (200~300mesh) by

elution with cyclohexane-ethyl acetate and silica gel GF_{254} used for analytical TLC was purchased from the Aldrich Chemical Company. Detection was effected by spraying the plates with 5% ethanolic H_2SO_4 (followed by heating at 110°C for 10 min.) or by direct UV irradiation of the plate.

α^4, α^5-*O*-isopropylidene-6-fluoropyrodoxol (2) The suspension of 6-fluoro-pyrodoxol **1** (2.0g, 10.68mmol) in anhydrous acetone (60mL) containing 2% concentrate H_2SO_4 was stirred for 4~5h, at the end of which time TLC (4:1 cyclohexane-EtOAc) indicated that the reaction was complete, then cold saturated Na_2CO_3 solution added with vigorous stirring up to pH between 8~9, the precipitate was filtered off, concentration of the reaction mixture under reduced pressure followed by purification on flash silica gel column with 4:1 cyclohexane-EtOAc as the eluent furnished the acetonide **2** (0.64g, 26%) as a syrup, R_f 0.34(4:1 cyclohexane-EtOAc), δ_H: 7.45(1H, s, HO-3), 5.03(2H, s, CH_2-5), 4.57(2H, s, CH_2-4), 2.33(3H, s, CH_3-2), 1.55(6H, s, 2×CH_3)ppm; δ_C: 154.49(s, Py-C), 152.20(s, Py-C), 144.14(d, $J_{F\text{-}C}$=14.5Hz, Py-C), 131.37(d, $J_{F\text{-}C}$=3.8Hz, Py-C), 114.01(d, $J_{F\text{-}C}$=32.9Hz, Py-C), 99.51(s, **C**Me_2), 59.04(d, $J_{F\text{-}C}$=3.8Hz, CH_2-5), 54.51(s, CH_2-4), 31.62(s, C(**C**H_3)$_2$), 17.58(s, CH_3-2)ppm.

3-*O*-Acetyl-α^4, α^5-*O*-isopropylidene-6-fluoropyrodoxol (3) A solution of **2** (0.62g, 2.72mmol) in pyridine (12mL) was treated with acetic anhydride (6mL). After being stirred from 0°C to r.t. for overnight, the reaction mixture was coevaporated with toluene under reduced pressure and the residue purified by flash silica gel column chromatography (eluent 5:1 cyclohexane-EtOAc) to give **3** (0.70g, 94%) as a syrup, R_f 0.45(5:1 cyclohexane-EtOAc), δ_H: 5.05(2H, s, CH_2-5), 4.61(2H, s, CH_2-4), 2.39(3H, s, CH_3-2), 2.08(3H, s, CH_3CO), 1.57(6H, s, 2×CH_3)ppm; δ_C: 170.02(s, CH_3**C**O), 154.67(s, Py-C), 152.31(s, Py-C), 144.35(d, $J_{F\text{-}C}$=14.7Hz, Py-C), 131.47(d, $J_{F\text{-}C}$=4.0Hz, Py-C), 114.18(d, $J_{F\text{-}C}$=33.05Hz, Py-C), 100.26(s, **C**Me_2), 59.24(d, $J_{F\text{-}C}$=3.9Hz, CH_2-5), 54.62(s, CH_2-4), 31.78(s, C(**C**H_3)$_2$), 21.53(s, **C**H_3CO), 17.62(s, CH_3-2)ppm.

3-*O*-Acetyl-6-fluoropyrodoxol (4) Compound **3** (0.68g, 2.52mmol) was dissolved in a solution of CH_2Cl_2 (15mL), CF_3CO_2H (0.4mL, 90%) was added, the reaction mixture was stirred for 24h at reflux temperature until TLC showed the compound **3** disappear, cooled to 0°C, and neutralized with $NaHCO_3$ up to pH between 7~8, the precipitate was filtered off, the mixture was evaporated, and the residue was

purified by silica gel column chromatography (10:1 $CHCl_3$-MeOH) to give compound **4** (144.0mg, 30%) as a syrup, R_f 0.15(10:1 $CHCl_3$-MeOH), δ_H: 5.77(1H, t, $J_{H\text{-}5,HO\text{-}5}$=4.9Hz, HO-5, exchangeable with D_2O), 5.10(1H, t, $J_{H\text{-}4,HO\text{-}4}$=5.2Hz, HO-4, exchangeable with D_2O), 4.78(2H, d, $J_{H\text{-}5,HO\text{-}5}$=4.9Hz, CH_2-5), 4.47(2H, d, $J_{H\text{-}4,HO\text{-}4}$=5.2Hz, CH_2-4), 2.30(3H, s, CH_3-2), 2.10(3H, s, CH_3CO)ppm; δ_C: 170.12(s, CH_3CO), 154.91(s, Py-C), 152.62(s, Py-C), 145.19(d, $J_{F\text{-}C}$=14.5Hz, Py-C), 131.50(d, $J_{F\text{-}C}$=3.0Hz, Py-C), 113.74(d, $J_{F\text{-}C}$=32.0Hz, Py-C), 57.77(d, $J_{F\text{-}C}$=3.0Hz, CH_2-5), 54.02(s, CH_2-4), 21.53(s, CH_3CO), 19.20(s, CH_3-2)ppm.

***α*^{4}-*O*-Acetyl-6-fluoropyrodoxol** (96.0mg, 20%), syrup, R_f 0.20(10:1 $CHCl_3$-MeOH), δ_H: 8.85(1H, s, HO-3, exchangeable with D_2O), 5.80(1H, t, $J_{H\text{-}5,HO\text{-}5}$=4.8Hz, HO-5, exchangeable with D_2O), 4.79(2H, d, $J_{H\text{-}5,HO\text{-}5}$=4.8Hz, CH_2-5), 4.58(2H, s, CH_2-4), 2.29(3H, s, CH_3-2), 2.12(3H, s, CH_3CO)ppm; δ_C: 170.71(s, CH_3CO), 154.95(s, Py-C), 152.66(s, Py-C), 145.23(d, $J_{F\text{-}C}$=14.7Hz, Py-C), 131.56(d, $J_{F\text{-}C}$=3.3Hz, Py-C), 113.77(d, $J_{F\text{-}C}$=32.5Hz, Py-C), 57.86(d, $J_{F\text{-}C}$=3.2Hz, CH_2-5), 55.45(s, CH_2-4), 21.73(s, CH_3CO), 18.30(s, CH_3-2)ppm.

***α*^{5}-*O*-Acetyl-6-fluoropyrodoxol** (72.0mg, 15%), syrup, R_f 0.25(10:1 $CHCl_3$-MeOH), δ_H: 8.65(1H, s, HO-3, exchangeable with D_2O), 5.14(1H, t, $J_{H\text{-}4,HO\text{-}4}$=5.0Hz, HO-4, exchangeable with D_2O), 4.86(2H, s, CH_2-5), 4.49(2H, d, $J_{H\text{-}4,HO\text{-}4}$=5.0Hz, CH_2-4), 2.27(3H, s, CH_3-2), 2.15(3H, s, CH_3CO)ppm; δ_C: 170.80(s, CH_3CO), 154.88(s, Py-C), 152.72(s, Py-C), 145.33(d, $J_{F\text{-}C}$=14.1Hz, Py-C), 131.76(d, $J_{F\text{-}C}$=3.5Hz, Py-C), 113.82(d, $J_{F\text{-}C}$=32.8Hz, Py-C), 58.12(d, $J_{F\text{-}C}$=3.6Hz, CH_2-5), 55.52(s, CH_2-4), 21.85(s, CH_3CO), 18.18(s, CH_3-2)ppm.

3-*O*-Acetyl-*α*^{4}, *α*^{5}-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyrodoxol (8), 3-*O*-acetyl-*α*^{4}, *α*^{5}-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-fluoropyrodoxol (9) and 3-*O*-acetyl-*α*^{4}, *α*^{5}-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyrodoxol (10) A solution of 2,3,4,6-tetra-*O*-acetyl-*α*-D-glucopyranosyl bromide (**5**) or 2,3,4,6-tetra-*O*-acetyl-*α*-D-galactopyranosyl bromide (**6**) and 2,3,4,6-tetra-*O*-acetyl-*α*-D-mannopyranosyl bromide (**7**) (0.54g, 1.29mmol, 1.1 equiv.) in anhydrous CH_2Cl_2 (5mL) was added dropwise into the solution of 3-*O*-acetyl-6-fluoropyrodoxol **4** (135mg, 0.60mmol) and $Hg(CN)_2$

(363.6mg, 1.44mmol) as a promoter in dry CH_2Cl_2 (5mL) containing powdered molecular sieves (4Å, 1.0g) with vigorous stirring at r.t. under an argon atmosphere in the dark for 12h. The mixture was diluted with CH_2Cl_2 (15mL), filtered through Celite, washed with water, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified on a silica gel column (1:2 cyclohexane-EtOAc) to yield the title compound **8**, **9** and **10**, respectively.

3-*O*-acetyl-α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyrodoxol **8** (0.48g, 90%), syrup, R_f 0.41(1:2 cyclohexane-EtOAc), δ_H: 5.34(1H, d, $J_{1',2'}$=8.0Hz, H-1'), 5.38(1H, d, $J_{1'',2''}$=8.0Hz, H-1''), 5.12(2H, dd, $J_{2',3'}$= $J_{2'',3''}$=7.2Hz, H-2',H-2''), 4.41(2H, dd, $J_{3',4'}$=$J_{3'',4''}$=3.2Hz, H-3', H-3''), 4.81(2H, dd, $J_{4',5'}$=$J_{4'',5''}$=3.6 Hz, H-4', H-4''), 3.92(2H, m, H-5', H-5''), 4.78(2H, dd, $J_{5',6a'}$=$J_{5'',6a''}$=2.8Hz, $J_{6a',6b'}$= $J_{6a'',6b''}$=10.0Hz, H-6a', H-6a''), 4.06(2H, dd, $J_{5',6b'}$=$J_{5'',6b''}$=3.2Hz, H-6b', H-6b''), 4.53(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=10.4Hz, CH_2-4a), 4.46(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=10.4Hz, CH_2-4b), 4.55(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=11.2Hz, CH_2-5a), 4.49(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=11.2Hz, CH_2-5b), 2.34(3H, s, CH_3-2), 2.11(3H, s, CH_3CO-3), 1.98, 1.97, 1.96, 1.95, 1.94, 1.93, 1.92, 1.91(24H, 8s, 8×CH_3CO)ppm; δ_C: 170.83, 170.73, 170.56, 170.28, 170.20, 170.17, 169.94, 169.82, 169.75(9×CH_3**C**O), 159.90(s, Py-C), 157.56(s, Py-C), 152.12(d, $J_{F\text{-}C}$= 16.1Hz, Py-C), 138.40(d, $J_{F\text{-}C}$=11.4Hz, Py-C), 117.48(d, $J_{F\text{-}C}$=32.0Hz, Py-C), 100.28(s, C-1'), 100.46(s, C-1''), 71.48(s, C-2', C-2''), 72.10(s, C-3'), 72.26(s, C-3''), 68.43(s, C-4'), 68.58(s, C-4''), 72.88(s, C-5'), 72.96(s, C-5''), 61.68(s, C-6'), 62.00(s, C-6''), 60.89(s, CH_2-4), 61.22(s, CH_2-5), 22.56(s, **C**H_3CO-3), 20.90, 20.88, 20.85, 20.80, 20.74, 20.73, 20.60, 20.59(8s, 8×**C**H_3CO), 19.41(s, CH_3-3)ppm.

3-*O*-acetyl-α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galacopyranosyl)-6-fluoropyrodoxol **9** (0.46g, 86%), syrup, R_f 0.30(2:3 cyclohexane-EtOAc), δ_H: 4.42(1H, d, $J_{1',2'}$=7.6Hz, H-1'), 4.51(1H, d, $J_{1'',2''}$=8.0Hz, H-1''), 5.12(2H, dd, $J_{2',3'}$= $J_{2'',3''}$=8.4Hz, H-2',H-2''), 4.97(1H, dd, $J_{3',4'}$=4.0Hz, H-3'), 4.99(1H, dd, $J_{3'',4''}$=3.6Hz, H-3''), 5.36(2H, dd, $J_{4',5'}$=$J_{4'',5''}$=2.8Hz, H-4', H-4''), 4.12(2H, m, H-5', H-5''), 4.07(2H, dd, $J_{5',6a'}$= $J_{5'',6a''}$=3.2Hz, $J_{6a',6b'}$=$J_{6a'',6b''}$=10.0Hz, H-6a', H-6a''), 3.91(2H, dd, $J_{5',6b'}$= $J_{5'',6b''}$=7.2Hz, H-6b', H-6b''), 4.76(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=18.4Hz, CH_2-4a), 4.73(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=18.4Hz, CH_2-4b), 4.92(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=12.4Hz, CH_2-5a), 4.69(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=12.4Hz, CH_2-5b), 2.33(3H, s, CH_3-2), 2.30(3H, s, CH_3CO-3), 2.12, 2.11, 2.04, 2.03, 2.01, 1.94, 1.93, 1.92(24H, 8s, 8×CH_3CO)ppm; δ_C: 170.67, 170.60, 170.53, 170.43,

170.40, 170.35, 170.27, 170.24, 170.16(9×CH_3**C**O), 159.95(s, Py-C), 157.58(s, Py-C), 151.43(d, $J_{F\text{-}C}$=16.1Hz, Py-C), 142.59(d, $J_{F\text{-}C}$=65.6Hz, Py-C), 116.28(d, $J_{F\text{-}C}$=32.1Hz, Py-C), 99.55(s, C-1'), 100.82(s, C-1''), 68.76(s, C-2'), 68.79(s, C-2''), 70.86(s, C-3'), 70.89(s, C-3''), 67.15(s, C-4', C-4''), 70.95(s, C-5'), 71.12(s, C-5''), 61.35(s, C-6'), 61.42(s, C-6''), 60.98(s, CH_2-4), 61.10(s, CH_2-5), 27.14(s, **C**H_3CO-3), 21.27, 20.94, 20.89, 20.85, 20.79, 20.77, 20.76, 20.58(8s, 8×**C**H_3CO), 19.45(s, CH_3-3)ppm.

3-*O*-acetyl-α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyrodoxol **10** (0.40g, 70%), syrup, R_f 0.45(1:2 cyclohexane-EtOAc), δ_H: 5.34(1H, d, $J_{1',2'}$=2.4Hz, H-1'), 5.35(1H, d, $J_{1'',2''}$=2.4Hz, H-1''), 5.32~3.90(16H, m, H-2',3',4',5',6',2'',3'',4'',5'',6'',CH_2-4,CH_2-5), 2.36(3H, s, CH_3-2), 2.33(3H, s, CH_3CO-3), 2.00, 1.99, 1.98, 1.97, 1.96, 1.95, 1.94, 1.93(24H, 8s, 8×CH_3CO)ppm; δ_C: 171.23, 171.15, 170.87, 170.83, 170.76, 170.64, 170.58, 170.44, 170.29(9×CH_3**C**O), 160.36(s, Py-C), 158.02(s, Py-C), 153.03(d, $J_{F\text{-}C}$=16.0Hz, Py-C), 145.40(d, $J_{F\text{-}C}$=4.6Hz, Py-C), 117.21(d, $J_{F\text{-}C}$=31.3Hz, Py-C), 98.32(s, C-1'), 98.99(s, C-1''), 72.54~56.46(12C, C-2',3',4',5',6',2'',3'',4'',5'',6'',CH_2-4,CH_2-5), 21.21, 20.92, 20.90, 20.89, 20.87, 20.82, 20.78, 20.76, 20.70(9s, 9×**C**H_3CO), 18.32(s, CH_3-3)ppm.

α^4, α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyrodoxol (T_1), α^4, α^5-di-*O*-(β-D-galacopyranosyl)-6-fluoropyrodoxol (T_2) and α^4, α^5-di-*O*-(α-D-mannopyranosyl)-6-fluoropyrodoxol (T_3) A solution of **8** or **9** and **10** (0.4g) in anhydrous MeOH(10mL) containing 0.5M NH_3 was vigorously stirred from 0°C to room temperature for overnight until TLC showed that the reaction complete, evaporated to dryness *in vacuo*. Chromatography of the crude syrup on silica gel with ethyl acetate-methanol (9:1) afforded **T_1~T_3**.

α^4, α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyrodoxol **T_1** (0.22g, 97%), syrup, R_f 0.25(1:4 MeOH-EtOAc), δ_H: 7.56(1H, s, HO-3, exchangeable with D_2O), 5.10(2H, d, $J_{1',2'}$=10.0Hz, H-1', H-1''), 4.80(2H, dd, $J_{2',3'}$=$J_{2'',3''}$=8.0Hz, H-2',H-2''), 4.67(2H, dd, $J_{3',4'}$=$J_{3'',4''}$=3.6Hz, H-3', H-3''), 4.90(2H, dd, $J_{4',5'}$=$J_{4'',5''}$=3.4Hz, H-4', H-4''), 3.61(2H, m, H-5', H-5''), 3.50(2H, dd, $J_{5',6a'}$=$J_{5'',6a''}$=4.7Hz, $J_{6a',6b'}$=$J_{6a'',6b''}$=11.3Hz, H-6a', H-6a''), 3.28(2H, dd, $J_{5',6b'}$=$J_{5'',6b''}$=5.8Hz, H-6b', H-6b''), 4.21(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=6.6Hz, CH_2-4a), 4.26(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=6.6Hz, CH_2-5a), 4.15(2H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=$J_{CH2\text{-}5a,CH2\text{-}}$

$_{5b}$=6.6Hz, CH_2-4b, CH_2-5b), 4.08~3.75(8H, br, HO-2',3',4',6',2'',3'',4'',6'', exchangeable with D_2O), 2.38(3H, s, CH_3-2)ppm; δ_C: 156.26(s, Py-C), 153.67(s, Py-C), 148.37(d, J_{F-C}=3.7Hz, Py-C), 145.45(d, J_{F-C}=14.9Hz, Py-C), 136.73(d, J_{F-C}=3.5Hz, Py-C), 102.95(s, C-1'), 103.56(s, C-1''), 71.04(s, C-2'), 70.80(s, C-2''), 73.64(s, C-3'), 73.75(s, C-3''), 68.28(s, C-4'), 68.38(s, C-4''), 75.32(s, C-5'), 75.92(s, C-5''), 60.66(s, C-6'), 60.86(s, C-6''), 60.25(s, CH_2-4), 60.38(s, CH_2-5), 19.75(s, CH_3-3)ppm.

α^4, α^5-di-*O*-(β-D-galacopyranosyl)-6-fluoropyrodoxol **T_2** (0.21g, 94%), foam solid, R_f 0. 28(1:4 MeOH-EtOAc), δ_H: 7.40(1H, s, HO-3, exchangeable with D_2O), 4.96 (2H, d, $J_{1',2'}$=10.8Hz, H-1', H-1''), 4.73(2H, dd, $J_{2',3'}$= $J_{2'',3''}$=8.4Hz, H-2',H-2''), 4.60(2H, dd, $J_{3',4'}$=$J_{3'',4''}$=3.8Hz, H-3', H-3''), 4.88(2H, dd, $J_{4',5'}$=$J_{4'',5''}$=3.6Hz, H-4', H-4''), 3.61(2H, m, H-5', H-5''), 3.47(2H, dd, $J_{5',6a'}$=$J_{5'',6a''}$=4.8Hz, $J_{6a',6b'}$=$J_{6a'',6b''}$=11.2Hz, H-6a', H-6a''), 3.23(2H, dd, $J_{5',6b'}$= $J_{5'',6b''}$=5.6Hz, H-6b', H-6b''), 4.18(1H, d, $J_{CH2-4a,CH2-4b}$=6.4Hz, CH_2-4a), 4.22(1H, d, $J_{CH2-4a,CH2-4b}$=6.4Hz, CH_2-5a), 4.13(2H, d, $J_{CH2-4a,CH2-4b}$=$J_{CH2-5a,CH2-5b}$=6.4Hz, CH_2-4b, CH_2-5b), 4.10~3.70(8H, br, HO-2',3',4',6',2'',3'',4'', 6'', exchangeable with D_2O), 2.36(3H, s, CH_3-2)ppm; δ_C: 155.39(s, Py-C), 153.13(s, Py-C), 147.87(d, J_{F-C}=3.9Hz, Py-C), 144.65(d, J_{F-C}=14.5Hz, Py-C), 137.36(d, J_{F-C}=3.8Hz, Py-C), 103.05(s, C-1'), 103.38(s, C-1''), 70.54(s, C-2'), 70.60(s, C-2''), 73.46(s, C-3'), 73.57(s, C-3''), 68.20(s, C-4'), 68.32(s, C-4''), 75.23(s, C-5'), 75.29(s, C-5''), 60.62(s, C-6'), 60.66(s, C-6''), 60.20(s, CH_2-4), 60.30(s, CH_2-5), 19.55(s, CH_3-3)ppm.

α^4, α^5-di-*O*-(α-D-mannopyranosyl)-6-fluoropyrodoxol **T_3** (0.20g, 90%), foam solid, R_f 0.30(1:4 MeOH-EtOAc), δ_H: 7.36(1H, s, HO-3, exchangeable with D_2O), 5.18 (2H, d, $J_{1',2'}$=2.8Hz, H-1', H-1''), 5.00~3.60(16H, m, H-2',3',4',5',6',2'',3'',4'',5'',6'', CH_2-4,CH_2-5), 4.15~3.76(8H, br, HO-2',3',4',6',2'',3'',4'', 6'', exchangeable with D_2O), 2.34(3H, s, CH_3-2)ppm; δ_C: 155.29(s, Py-C), 152.98(s, Py-C), 147.37(d, J_{F-C}=3.6Hz, Py-C), 145.46(d, J_{F-C}=14.0Hz, Py-C), 137.16(d, J_{F-C}=3.5Hz, Py-C), 100.25(s, C-1'), 100.39(s, C-1''), 70.41(s, C-2', C-2''), 73.40(s, C-3'), 73.35(s, C-3''), 67.80(s, C-4'), 67.91(s, C-4''), 74.53(s, C-5'), 74.69(s, C-5''), 60.51(s, C-6'), 60.64(s, C-6''), 60.02(s, CH_2-4), 60.10(s, CH_2-5), 19.32(s, CH_3-3)ppm.

3-*O*-benzyl-α^4, α^5-*O*-isopropylidene-6-fluoropyrodoxol 11 To a solution of α^4, α^5-*O*-isopropylidene-6-fluoropyrodoxol **2** (0.5g, 2.20mmol) in anhydrous DMF (20mL), NaH (60%) (0.11g, 2.60mmol) was added. The mixture was stirred at r.t. for 30

min, and then benzyl bromide (0.45g, 2.60mmol) was added dropwise. The reaction was completed after 2 h, as indicated by TLC. Drops of MeOH were added to decompose excess NaH, and then the mixture was diluted with CH_2Cl_2 (30mL) and washed with water. The organic extracts were dried (Na_2SO_4) and evaporated *in vacuo*, the residue was chromatographied on silica gel with cyclohexane-EtOAc (5:1) to afford the desired compound **11** (0.66g, 94%), as a syrup, R_f 0.48(5:1 cyclohexane-EtOAc), δ_H: 7.40(5H, m, Ar-H), 5.10(2H, s, CH_2-5), 4.92(2H, s, $PhCH_2$), 4.80(2H, s, CH_2-4), 2.41(3H, s, CH_3-2), 1.65, 1.62(6H, 2s, 2×CH_3)ppm; δ_C: 158.83(s, Py-C), 156.28(s, Py-C), 151.38(d, J_{F-C}=14.4Hz, Py-C), 147.37(d, J_{F-C}=4.4Hz, Py-C), 136.43, 128.99, 128.89, 128.65(Ph-C), 120.46(d, J_{F-C}=32.05Hz, Py-C), 100.34(s, **C**Me_2), 59.64(d, J_{F-C}=3.0Hz, CH_2-5), 56.04(s, $PhCH_2$), 54.78(s, CH_2-4), 32.01(s, C(**C**H_3)$_2$), 18.65(s, CH_3-2)ppm.

3-*O*-Benzyl-6-fluoropyrodoxol 12 Compound **11** (0.65g, 2.05mmol) was dissolved in a solution of MeOH(15mL)-CF_3CO_2H (0.5mL, 90%), and the reaction mixture was stirred for ~6h at reflux temperature until TLC showed the compound **11** disappear, cooled to 0°C, and neutralized with $NaHCO_3$ up to pH between 7~8, the precipitate was filtered off, the mixture was evaporated, and the residue was purified by silica gel column chromatography (1:2 cyclohexane-EtOAc) to give compound **12** (0.50g, 88%) as a white crystalline, R_f 0.38(1:2 cyclohexane-EtOAc), δ_H: 7.39(5H, m, Ar-H), 4.90(2H, s, $PhCH_2$), 4.75(2H, d, $J_{H-5,HO-5}$=5.4Hz, CH_2-5), 4.72(2H, d, $J_{H-4,HO-4}$=6.0Hz, CH_2-4), 3.57(1H, t, $J_{H-5,HO-5}$=5.4Hz, HO-5, exchangeable with D_2O), 3.49(1H, t, $J_{H-4,HO-4}$=6.0Hz, HO-4, exchangeable with D_2O), 2.44(3H, s, CH_3-2)ppm; δ_C: 157.38(s, Py-C), 155.82(s, Py-C), 149.55(d, J_{F-C}=4.7Hz, Py-C), 146.97(d, J_{F-C}=4.0Hz, Py-C), 119.09(d, J_{F-C}=31.2Hz, Py-C), 136.33, 128.96, 128.88, 128.57(Ph-C), 55.99(s, $PhCH_2$, CH_2-4), 56.76(s, CH_2-5), 19.31(s, CH_3-2)ppm.

An alternative synthesis of **12** from **1** directly by phase transfer catalysis was described as follows; To a well stirred CH_2Cl_2(10mL)-H_2O(10mL) biphasic mixture (pH 10~11) of **1** (0.5g, 2.67mmol) and TBAB (0.1g, 0.31mmol) as the phase-transfer catalyst, a solution of benzyl bromide (0.51g, 2.94mmol, 1.1equiv.) in CH_2Cl_2 (10mL) was added dropwise over a period of 4~5h, while the reaction temperature was maintained at 50°C, and the stirring continued for an additional hour, whereupon the products were extracted with CH_2Cl_2 (4×20mL), washed free of alkali, dried(Na_2SO_4), and concentrated, the

residue was purified by column chromatography on silica gel with 1:2 cyclohexane-EtOAc as the eluent to afford **12** (0.56g, 76%) as a white crystalline, which is identical in all respects to the product obtained in the above procedure.

3-*O*-Benzyl-α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyrodoxol (13), 3-*O*-Benzyl-α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-fluoropyrodoxol (14) and 3-*O*-Benzyl-α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyrodoxol (15) Condensation of **12** (0.10g, 0.36mmol) with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**5**) or 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**6**) and 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide (**7**) (0.33g, 0.79mmol, 1.1equiv.) in dry CH_2Cl_2 (5mL) with $Hg(CN)_2$ (0.20g, 0.80mmol) as a promoter, according to the procedures described for the preparation of **8**~**10**, furnished the desired compounds **13**~**15**, respectively.

3-*O*-Benzyl-α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyrodoxol **13** (0.32g, 95%), syrup, R_f 0.35(3:2 cyclohexane-EtOAc), δ_H: 7.41(5H, m, Ar-H), 5.36(1H, d, $J_{1',2'}$=8.2Hz, H-1'), 5.41(1H, d, $J_{1'',2''}$=8.2Hz, H-1''), 5.14(2H, dd, $J_{2',3'}$=$J_{2'',3''}$=7.4Hz, H-2',H-2''), 4.45(2H, dd, $J_{3',4'}$=$J_{3'',4''}$=3.3Hz, H-3', H-3''), 4.84(2H, dd, $J_{4',5'}$=$J_{4'',5''}$=3.8Hz, H-4', H-4''), 3.96(2H, m, H-5', H-5''), 4.80(2H, dd, $J_{5',6a'}$=$J_{5'',6a''}$=2.6Hz, $J_{6a',6b'}$=$J_{6a'',6b''}$=10.1Hz, H-6a', H-6a''), 4.10(2H, dd, $J_{5',6b'}$=$J_{5'',6b''}$ =3.0Hz, H-6b', H-6b''), 4.94(2H, s, $PhCH_2$), 4.55(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=10.4Hz, CH_2-4a), 4.48(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=10.4Hz, CH_2-4b), 4.60(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=11.0Hz, CH_2-5a), 4.52(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=11.0Hz, CH_2-5b), 2.37(3H, s, CH_3-2), 2.00, 1.99, 1.98, 1.97, 1.96, 1.95, 1.94, 1.93(24H, 8s, 8×CH_3CO)ppm; δ_C: 170.84, 170.76, 170.31, 170.29, 170.26, 169.95, 169.92, 169.84(8×CH_3**C**O), 158.57(s, Py-C), 156.23(s, Py-C), 152.18(d, $J_{F\text{-}C}$=14.5Hz, Py-C), 142.64(d, $J_{F\text{-}C}$=4.6Hz, Py-C), 116.20(d, $J_{F\text{-}C}$=32.0Hz, Py-C), 136.37, 129.00, 128.94, 128.87, 128.16, 127.77(Ph-C), 100.23(s, C-1'), 100.41(s, C-1''), 71.41(s, C-2', C-2''), 72.08(s, C-3'), 72.19(s, C-3''), 68.34(s, C-4'), 68.51(s, C-4''), 72.86(s, C-5'), 72.93(s, C-5''), 61.86(s, C-6'), 61.98(s, C-6''), 60.98(s, CH_2-4), 61.28(s, CH_2-5), 20.88, 20.85, 20.82, 20.75, 20.73, 20.60, 20.59, 20.58(8s, 8×**C**H_3CO), 19.43(s, CH_3-3)ppm.

3-*O*-Benzyl-α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-fluoropyrodoxol **14** (0.31g, 93%), syrup, R_f 0.38(3:2 cyclohexane-EtOAc), δ_H: 7.43(5H,

m, Ar-H), 4.46(1H, d, $J_{1',2'}$=7.8Hz, H-1'), 4.54(1H, d, $J_{1'',2''}$=8.0Hz, H-1''), 5.16(2H, dd, $J_{2',3'}$= $J_{2'',3''}$=8.0Hz, H-2',H-2''), 5.00(1H, dd, $J_{3',4'}$=4.2Hz, H-3'), 5.04(1H, dd, $J_{3'',4''}$=3.6Hz, H-3''), 5.40(2H, dd, $J_{4',5'}$=$J_{4'',5''}$=3.0Hz, H-4', H-4''), 4.16(2H, m, H-5', H-5''), 4.10(2H, dd, $J_{5',6a'}$= $J_{5'',6a''}$=3.2Hz, $J_{6a',6b'}$=$J_{6a'',6b''}$=10.6Hz, H-6a', H-6a''), 3.98(2H, dd, $J_{5',6b'}$= $J_{5'',6b''}$=7.0Hz, H-6b', H-6b''), 4.96(2H, s, $PhCH_2$), 4.78(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=18.0Hz, CH_2-4a), 4.75(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=18.0Hz, CH_2-4b), 4.95(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=12.0Hz, CH_2-5a), 4.72(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=12.0Hz, CH_2-5b), 2.38(3H, s, CH_3-2), 2.10, 2.08, 2.06, 2.04, 2.03, 1.98, 1.95, 1.94(24H, 8s, 8×CH_3CO)ppm; δ_C: 170.54, 170.39, 170.32, 170.27, 169.66, 169.63, 169.58, 169.54(8×CH_3**C**O), 158.31(s, Py-C), 156.74(s, Py-C), 152.17(d, $J_{F\text{-}C}$=14.5Hz, Py-C), 142.76(s, Py-C), 116.30(d, $J_{F\text{-}C}$=21.3Hz, Py-C), 136.40, 129.53, 128.67, 128.46, 128.42, 127.64(Ph-C), 100.27(s, C-1'), 101.22(s, C-1''), 68.45(s, C-2'), 68.48(s, C-2''), 70.52(s, C-3'), 70.62(s, C-3''), 66.57(s, C-4'), 66.71(s, C-4''), 71.45(s, C-5'), 71.56(s, C-5''), 62.35(s, C-6'), 62.75(s, C-6''), 61.22(s, CH_2-4), 61.38(s, CH_2-5), 21.30, 20.25, 21.13, 20.43, 20.39, 20.32, 20.30, 20.27(8s, 8×**C**H_3CO), 19.02(s, CH_3-3)ppm.

3-*O*-Benzyl-α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyrodoxol **15** (0.30g, 90%), syrup, R_f 0.40(3:2 cyclohexane-EtOAc), δ_H: 7.38(5H, m, Ar-H), 5.38(1H, d, $J_{1',2'}$=2.6Hz, H-1'), 5.41(1H, d, $J_{1'',2''}$=2.6Hz, H-1''), 5.36~3.95 (18H, m, H-2',3',4',5',6',2'',3'',4'',5'',6'',$PhCH_2$,CH_2-4,CH_2-5), 2.38(3H, s, CH_3-2), 2.02, 2.00, 1.99, 1.98, 1.97, 1.96, 1.95, 1.94(24H, 8s, 8×CH_3CO)ppm; δ_C: 171.25, 171.18, 170.89, 170.85, 170.78, 170.66, 170.60, 170.48(8×CH_3**C**O), 159.63(s, Py-C), 158.24(s, Py-C), 153.28(d, $J_{F\text{-}C}$=15.8Hz, Py-C), 145.48(d, $J_{F\text{-}C}$=4.8Hz, Py-C), 118.10(d, $J_{F\text{-}C}$=31.0Hz, Py-C), 98.42(s, C-1'), 100.03(s, C-1''), 72.60~56.54(13C, C-2',3',4',5',6',2'',3'',4'',5'',6'',$PhCH_2$,CH_2-4,CH_2-5), 21.23, 20.94, 20.92, 20.90, 20.88, 20.86, 20.84, 20.80, 20.78(8s, 8×**C**H_3CO), 18.37(s, CH_3-3)ppm.

α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyrodoxol (16), α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-fluoropyrodoxol (17) and α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyrodoxol (18) A mixture of **13~15** (0.29g, 0.3mmol) and Pd-C (5%, 300mg) in

MeOH (40mL) was stirred for 24h at r.t. under H_2 atmosphere. After filtration, the filtrate was evaporated to afford **16~18** in quantitative yields, respectively.

α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyrodoxol **16** (0.26g), syrup, R_f 0.28(3:2 cyclohexane-EtOAc), δ_H: 7.33(1H, s, HO-3, exchangeable with D_2O), 5.30(1H, d, $J_{1',2'}$=8.4Hz, H-1'), 5.35(1H, d, $J_{1'',2''}$=8.4Hz, H-1''), 5.09(2H, dd, $J_{2',3'}$=$J_{2'',3''}$=7.6Hz, H-2',H-2''), 4.35(2H, dd, $J_{3',4'}$=$J_{3'',4''}$=3.4Hz, H-3', H-3''), 4.80 (2H, dd, $J_{4',5'}$=$J_{4'',5''}$=3.6Hz, H-4', H-4''), 3.89(2H, m, H-5', H-5''), 4.77(2H, dd, $J_{5',6a'}$= $J_{5'',6a''}$=2.4Hz, $J_{6a',6b'}$=$J_{6a'',6b''}$=10.6Hz, H-6a', H-6a''), 4.05(2H, dd, $J_{5',6b'}$=$J_{5'',6b''}$ =3.2Hz, H-6b', H-6b''), 4.51(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=10.3Hz, CH_2-4a), 4.45(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$ =10.3Hz, CH_2-4b), 4.57(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=11.1Hz, CH_2-5a), 4.49(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$ =11.1Hz, CH_2-5b), 2.35(3H, s, CH_3-2), 1.99, 1.98, 1.97, 1.96, 1.95, 1.94, 1.93, 1.91(24H, 8s, 8×CH_3CO)ppm; δ_C: 170.82, 170.78, 170.65, 170.58, 170.46, 169.85, 169.82, 169.80(8×CH_3**C**O), 158.77(s, Py-C), 156.35(s, Py-C), 152.28(d, $J_{F\text{-}C}$=14.2Hz, Py-C), 142.69(d, $J_{F\text{-}C}$=4.8Hz, Py-C), 116.26(d, $J_{F\text{-}C}$=32.2Hz, Py-C), 100.35(s, C-1'), 100.54(s, C-1''), 71.37(s, C-2', C-2''), 72.18(s, C-3'), 72.29(s, C-3''), 68.38(s, C-4'), 68.56(s, C-4''), 72.83(s, C-5'), 72.88(s, C-5''), 61.82(s, C-6'), 61.89(s, C-6''), 60.90(s, CH_2-4), 61.19(s, CH_2-5), 20.85, 20.83, 20.82, 20.80, 20.78, 20.76, 20.73, 20.65(8s, 8×**C**H_3CO), 19.32(s, CH_3-3)ppm.

α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-fluoropyrodoxol **17** (0.26g), syrup, R_f 0.25(3:2 cyclohexane-EtOAc), δ_H: 7.30(1H, s, HO-3, exchangeable with D_2O), 4.39(1H, d, $J_{1',2'}$=8.0Hz, H-1'), 4.24(1H, d, $J_{1'',2''}$=8.0Hz, H-1''), 5.11(2H, dd, $J_{2',3'}$=$J_{2'',3''}$=8.0Hz, H-2',H-2''), 5.05(1H, dd, $J_{3',4'}$=4.4Hz, H-3'), 5.01(1H, dd, $J_{3'',4''}$=3.8Hz, H-3''), 5.32(2H, dd, $J_{4',5'}$=$J_{4'',5''}$=3.1Hz, H-4', H-4''), 4.11(2H, m, H-5', H-5''), 4.06(2H, dd, $J_{5',6a'}$= $J_{5'',6a''}$=3.2Hz, $J_{6a',6b'}$=$J_{6a'',6b''}$=10.8Hz, H-6a', H-6a''), 3.86(2H, dd, $J_{5',6b'}$= $J_{5'',6b''}$=7.1Hz, H-6b', H-6b''), 4.70(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=16.8Hz, CH_2-4a), 4.65(1H, d, $J_{CH2\text{-}4a,CH2\text{-}4b}$=16.8Hz, CH_2-4b), 4.59(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=12.6Hz, CH_2-5a), 4.42(1H, d, $J_{CH2\text{-}5a,CH2\text{-}5b}$=12.6Hz, CH_2-5b), 2.35(3H, s, CH_3-2), 2.11, 2.09, 2.07, 2.06, 2.04, 2.02, 2.00, 1.96(24H, 8s, 8×CH_3CO)ppm; δ_C: 170.55, 170.49, 170.38, 170.29, 169.76, 169.69, 169.59, 169.53(8×CH_3**C**O), 158.37(s, Py-C), 156.77(s, Py-C), 152.15(d, $J_{F\text{-}C}$=14.6Hz, Py-C), 142.73(s, Py-C), 116.33(d, $J_{F\text{-}C}$=21.4Hz, Py-C), 100.37(s, C-1'), 101.29(s, C-1''), 68.48(s, C-2'), 68.53(s, C-2''), 70.57(s, C-3'), 70.65(s, C-3''), 66.59(s,

C-4'), 66.75(s, C-4''), 71.49(s, C-5'), 71.61(s, C-5''), 62.39(s, C-6'), 62.83(s, C-6''), 61.31(s, CH_2-4), 61.43(s, CH_2-5), 21.33, 20.29, 20.18, 20.13, 20.09, 20.05, 20.02, 20.00(8s, 8×**C**H_3CO), 19.22(s, CH_3-3)ppm.

α^4, α^5-di-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyrodoxol **18** (0.26g), syrup, R_f 0.27(3:2 cyclohexane-EtOAc), δ_H: 7.33(1H, s, HO-3, exchangeable with D_2O), 5.33(1H, d, $J_{1',2'}$=2.7Hz, H-1'), 5.37(1H, d, $J_{1'',2''}$=2.7Hz, H-1''), 5.45~4.07 (16H, m, H-2',3',4',5',6',2'',3'',4'',5'',6'',CH_2-4,CH_2-5), 2.35(3H, s, CH_3-2), 2.01, 2.00, 1.99, 1.98, 1.97, 1.96, 1.95, 1.94(24H, 8s, 8×CH_3CO)ppm; δ_C: 171.33, 171.21, 170.85, 170.83, 170.76, 170.61, 170.56, 170.53(8×CH_3**C**O), 158.66(s, Py-C), 157.74(s, Py-C), 153.67(d, $J_{F\text{-}C}$=15.8Hz, Py-C), 145.68(d, $J_{F\text{-}C}$=4.6Hz, Py-C), 118.23(d, $J_{F\text{-}C}$=31.2Hz, Py-C), 98.67(s, C-1'), 100.33(s, C-1''), 72.8~56.56(12C, C-2',3',4',5',6',2'', 3'',4'',5'',6'', CH_2-4,CH_2-5), 20.99, 20.97, 20.93, 20.90, 20.88, 20.86, 20.84, 20.80(8s, 8×**C**H_3CO), 18.45(s, CH_3-3)ppm.

Treatment of **16** or **17** and **18** as described above for deacetylation to yield $\mathbf{T_1}$~$\mathbf{T_3}$, which are consistent with the products obtained from **8** or **9** and **10** in the above procedure in all physical and spectral data.

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